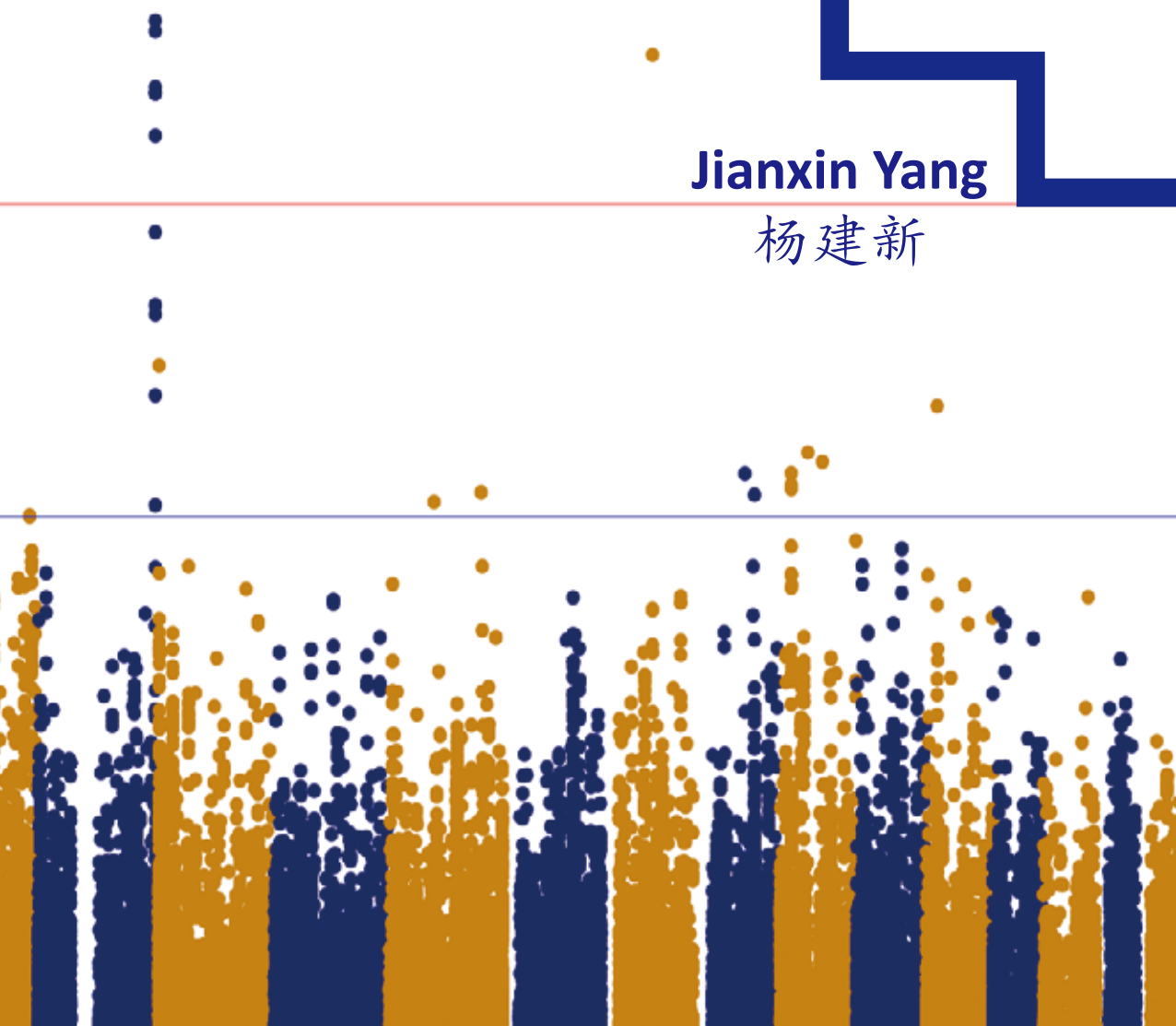
Molecular and genetic markers for the prediction of kidney transplant outcome

Yang, J.

MOLECULAR AND GENETIC MARKERS FOR THE PREDICTION OF KIDNEY TRANSPLANT OUTCOME

Jianxin Yang
杨建新



MOLECULAR AND GENETIC MARKERS FOR THE PREDICTION OF KIDNEY TRANSPLANT OUTCOME

Jianxin Yang
杨建新

Molecular and genetic markers for the prediction of kidney transplant outcome
Dissertation, University of Leiden, Leiden, the Netherlands

ISBN/EAN: 978-94-028-1244-2
Printed by: Ipskamp Printing
Author: Jianxin Yang

MOLECULAR AND GENETIC MARKERS FOR THE PREDICTION OF KIDNEY TRANSPLANT OUTCOME

Proefschrift

ter verkrijging van
de graad van Doctor aan de Universiteit Leiden,
op gezag van Rector Magnificus prof.mr. C.J.J.M. Stolker,
volgens besluit van het College voor Promoties

te verdedigen op woensdag 19 december 2018
klokke 10.00 uur

door

Jianxin Yang

geboren te Zhangjiakou, China
in 1985

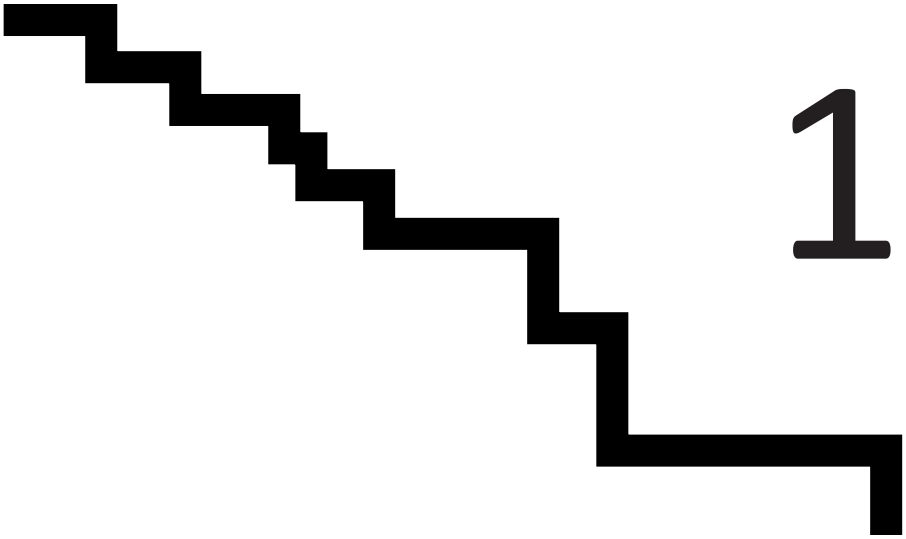
Promotor:	Prof. Dr. F.H.J. Claas
Co-promotor:	Dr. M. Eikmans
Leden commissie:	Prof. Dr. F. Koning Prof.Dr. MEJ Reinders Prof.Dr. S. Florquin (University of Amsterdam) Dr. H.G. Otten (University Medical Center Utrecht)

Financial support for the publication of this thesis was kindly provided by the University Libraries Leiden, National Reference Center for Histocompatibility Testing and the Nederlandse Transplantatie Vereniging.

TABLE OF CONTENTS

For My Family

General introduction



1

Content

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.

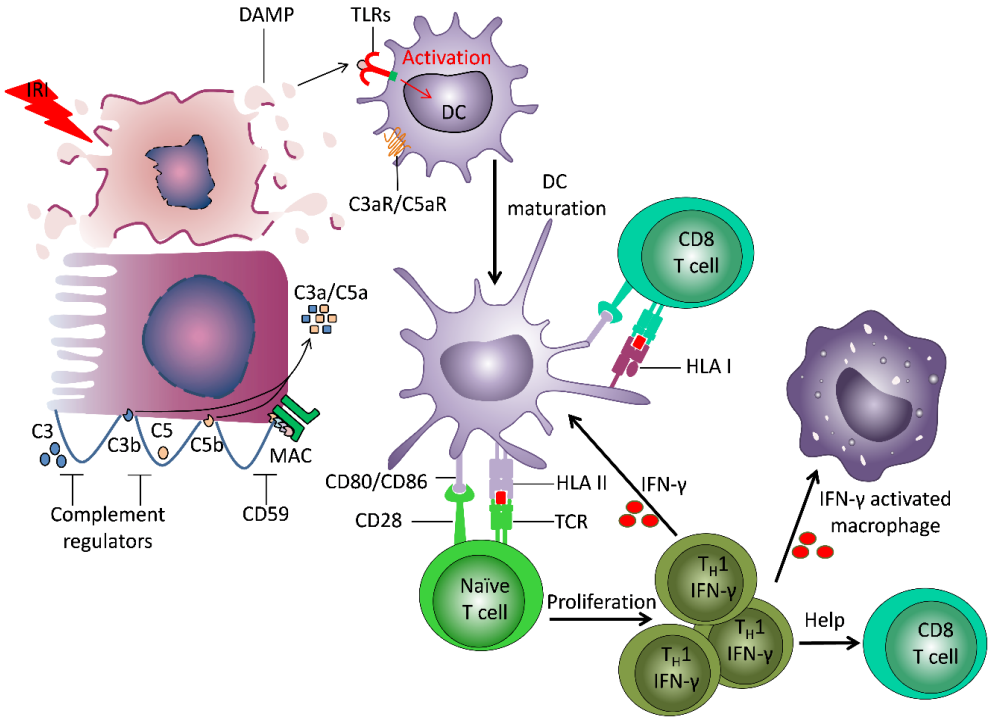


Figure 1. Danger signals in transplant immune activation. Ischemia reperfusion injury (IRI) leads to induction of necrosis of tubular cells and release of damage associated molecular patterns (DAMPs), which are normally hidden within intact cells. DAMPs binds to Toll-like receptors (TLRs) on dendritic 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.

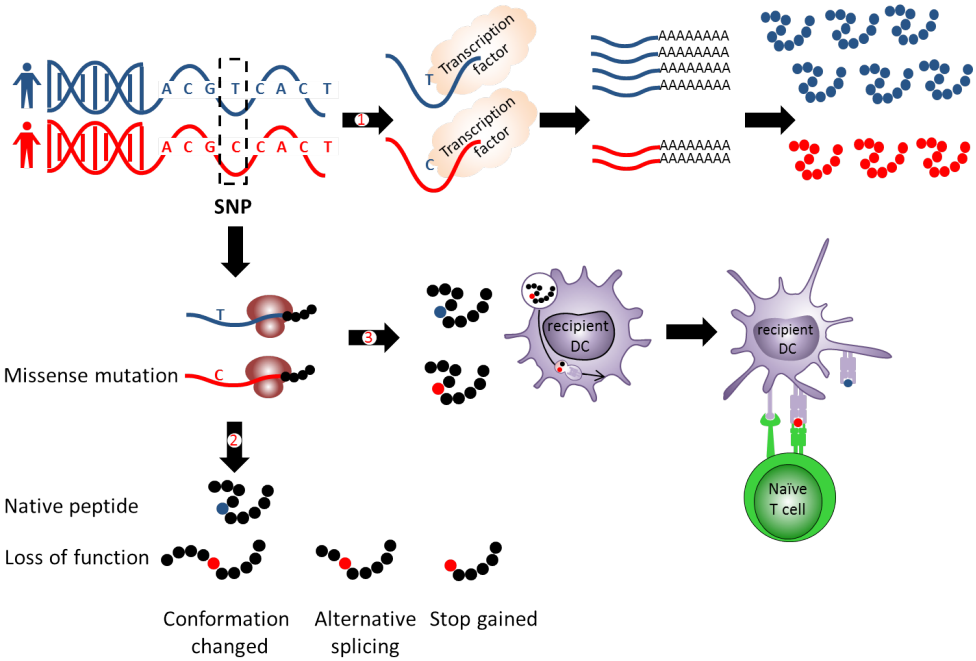


Figure 2. The role of single nucleotide polymorphism in the alloimmune response. SNPs may affect alloimmune responses through multiple mechanisms. Firstly, SNPs located in the promoter of genes may affect the binding of transcription factors, which subsequently alter gene expression of immune-related 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.

References

1. Levey AS, Coresh J. Chronic kidney disease. The lancet. 2012;379(9811):165-80.
2. Wolfe RA, Ashby VB, Milford EL, Ojo AO, Ettenger RE, Agodoa LY, et al. Comparison of mortality in all patients on dialysis, patients on dialysis awaiting transplantation, and recipients of a first cadaveric transplant. New England Journal of Medicine. 1999;341(23):1725-30.
3. Merrill JP, Murray JE, Harrison JH, Guild WR. Successful homotransplantation of the human kidney between identical twins. Journal of the American Medical Association. 1956;160(4):277-82.
4. Muntean A, Lucan M. Immunosuppression in kidney transplantation. Clujul Medical. 2013;86(3):177.
5. Pallardó Mateu LM, Sancho Calabuig A, Capdevila Plaza L, Franco Esteve A. Acute rejection and late renal transplant failure: risk factors and prognosis. Nephrology Dialysis Transplantation. 2004;19(suppl_3):iii38-iii42.
6. Thorsby E. A short history of HLA. HLA. 2009;74(2):101-16.
7. Sheldon T. jon van Rood. BMJ: British Medical Journal (Online). 2017;358.
8. Ting A, Morris P. Matching for B-cell antigens of the HLA-DR series in cadaver renal transplantation. The Lancet. 1978;311(8064):575-7.
9. Persijn G, Gabb B, Van Leeuwen A, Nagtegaal A, Hoogeboom J, Van Rood J. Matching for HLA antigens of A, B, and DR loci in renal transplantation by Eurotransplant. The Lancet. 1978;311(8077):1278-81.
10. Doxiadis, II, de Fijter JW, Mallat MJ, Haasnoot GW, Ringers J, Persijn GG, et al. Simpler and equitable allocation of kidneys from postmortem donors primarily based on full HLA-DR compatibility. Transplantation. 2007;83(9):1207-13.
11. Claas FH, Roelen DL, Dankers MK, Persijn GG, Doxiadis II. A critical appraisal of HLA matching in today's renal transplantation. Transplantation Reviews. 2004;18(2):96-102.
12. Humar A, Matas AJ, editors. Surgical complications after kidney transplantation. Seminars in dialysis; 2005: Wiley Online Library.
13. Yarlagadda SG, Coca SG, Formica Jr RN, Poggio ED, Parikh CR. Association between delayed graft function and allograft and patient survival: a systematic review and meta-analysis. Nephrol Dial Transpl. 2008;24(3):1039-47.
14. Ojo AO, Wolfe RA, Held PJ, Port FK, Schmouder RL. Delayed Graft Function: Risk Factors and Implications for Renal Allograft Survival1. Transplantation. 1997;63(7):968-74.
15. Giral-Classe M, Hourmant M, Cantarovich D, Dantal J, Blancho G, Daguin P, et al. Delayed graft function of more than six days strongly decreases long-term survival of transplanted kidneys. Kidney international. 1998;54(3):972-8.
16. Kosieradzki M, Rowiński W, editors. Ischemia/reperfusion injury in kidney transplantation: mechanisms and prevention. Transplantation proceedings; 2008: Elsevier.
17. Van Der Hoeven JA, Moshage H, Schuurs T, Nijboer M, Van Schilfgaarde R, Ploeg RJ. Brain death induces apoptosis in donor liver of the rat. Transplantation. 2003;76(8):1150-4.
18. van Der Hoeven JA, Molema G, Ter Horst GJ, Freund RL, Wiersema J, Van Schilfgaarde R, et al. Relationship between duration of brain death and hemodynamic (in) stability on progressive dysfunction and increased immunologic activation of donor kidneys. Kidney international. 2003;64(5):1874-82.
19. Jassem W, Koo DD, Muiesan P, Cerundolo L, Rela M, Fuggle SV, et al. Non-heart-beating versus cadaveric and living-donor livers: differences in inflammatory markers before transplantation. Transplantation. 2003;75(8):1386-90.
20. Nijboer WN, Schuurs TA, van der Hoeven JA, Fekken S, Wiersema-Buist J, Leuvenink HG, et al. Effect of brain death on gene expression and tissue activation in human donor kidneys. Transplantation. 2004;78(7):978-86.
21. Ishii KJ, Suzuki K, Coban C, Takeshita F, Itoh Y, Matoba H, et al. Genomic DNA released by dying cells induces the maturation of APCs. The Journal of Immunology. 2001;167(5):2602-7.
22. Ohashi K, Burkart V, Flohé S, Kolb H. Cutting edge: heat shock protein 60 is a putative endogenous ligand of the toll-like receptor-4 complex. The Journal of Immunology. 2000;164(2):558-61.
23. Park JS, Svetkauskaite D, He Q, Kim J-Y, Strassheim D, Ishizaka A, et al. Involvement of toll-like receptors 2 and 4 in cellular activation by high mobility group box 1 protein. Journal of Biological Chemistry. 2004;279(9):7370-7.
24. Nankivell BJ, Alexander SI. Rejection of the kidney allograft. New England Journal of Medicine. 2010;363(15):1451-62.
25. Williams GM, Hume DM, Hudson Jr RP, Morris PJ, Kano K, Milgrom F. Hyperacute renal-homograft

rejection in man. New England Journal of Medicine. 1968;279(12):611-8.
26. Starzl TE, Marchioro T, Holmes J, Hermann G, Brittain R, Stonington O, et al. Renal homografts in patients with major donor-recipient blood group incompatibilities. Surgery. 1964;55(2):195-200.
27. Colvin RB. Antibody-mediated renal allograft rejection: diagnosis and pathogenesis. Journal of the American Society of Nephrology : JASN. 2007;18(4):1046-56.
28. Patel R, Terasaki PI. Significance of the positive crossmatch test in kidney transplantation. New England Journal of Medicine. 1969;280(14):735-9.
29. Terasaki PI. Humoral theory of transplantation. American Journal of Transplantation. 2003;3(6):665-73.
30. Macedo C, Orkis E, Popescu I, Elinoff B, Zeevi A, Shapiro R, et al. Contribution of naive and memory T-cell populations to the human alloimmune response. American Journal of Transplantation. 2009;9(9):2057-66.
31. Wood KJ, Goto R. Mechanisms of rejection: current perspectives. Transplantation. 2012;93(1):1-10.
32. Smyth LA, Herrera OB, Golshayan D, Lombardi G, Lechler RI. A novel pathway of antigen presentation by dendritic and endothelial cells: Implications for allorecognition and infectious diseases. Transplantation. 2006;82:S15-S8.
33. Segerer S, Cui Y, Eitner F, Goodpaster T, Hudkins KL, Mack M, et al. Expression of chemokines and chemokine receptors during human renal transplant rejection. American Journal of Kidney Diseases. 2001;37(3):518-31.
34. Zuidwijk K, De Fijter JW, Mallat MJ, Eikmans M, Van Groningen MC, Goemaere NN, et al. Increased influx of myeloid dendritic cells during acute rejection is associated with interstitial fibrosis and tubular atrophy and predicts poor outcome. Kidney international. 2012;81(1):64-75.
35. Aubert O, Loupy A, Hidalgo L, van Huyen J-PD, Higgins S, Viglietti D, et al. Antibody-mediated rejection due to preexisting versus de novo donor-specific antibodies in kidney allograft recipients. Journal of the American Society of Nephrology. 2017;28(6):1912-23.
36. Einecke G, Sis B, Reeve J, Mengel M, Campbell P, Hidalgo L, et al. Antibody-mediated microcirculation injury is the major cause of late kidney transplant failure. American Journal of Transplantation. 2009;9(11):2520-31.
37. Wehmeier C, Amico P, Hirt-Minkowski P, Georgalis A, Höenger G, Menter T, et al. Acute rejection phenotypes in the current era of immunosuppression: a single-center analysis. Transplantation Direct. 2017;3(3).
38. Zou Y, Stastny P, Süsal C, Döhler B, Opelz G. Antibodies against MICA antigens and kidney-transplant rejection. New England Journal of Medicine. 2007;357(13):1293-300.
39. Truong LD, Barrios R, Adrogue HE, Gaber LW. Acute antibody-mediated rejection of renal transplant: pathogenetic and diagnostic considerations. Archives of pathology & laboratory medicine. 2007;131(8):1200-8.
40. Loupy A, Haas M, Solez K, Racusen L, Glotz D, Seron D, et al. The Banff 2015 kidney meeting report: current challenges in rejection classification and prospects for adopting molecular pathology. American Journal of Transplantation. 2017;17(1):28-41.
41. Solez K, Colvin R, Racusen L, Sis B, Halloran P, Birk P, et al. Banff'05 Meeting Report: differential diagnosis of chronic allograft injury and elimination of chronic allograft nephropathy ('CAN'). American Journal of Transplantation. 2007;7(3):518-26.
42. Otten H, Verhaar M, Borst H, Hené R, Van Zuilen A. Pretransplant donor-specific HLA class-I and-II antibodies are associated with an increased risk for kidney graft failure. American journal of transplantation. 2012;12(6):1618-23.
43. Worthington JE, Martin S, Al-Husseini DM, Dyer PA, Johnson RW. Posttransplantation production of donor HLA-specific antibodies as a predictor of renal transplant outcome1. Transplantation. 2003;75(7):1034-40.
44. Rule AD, Larson TS, Bergstralh EJ, Slezak JM, Jacobsen SJ, Cosio FG. Using serum creatinine to estimate glomerular filtration rate: accuracy in good health and in chronic kidney disease. Annals of internal medicine. 2004;141(12):929-37.
45. Solez K, Axelsen RA, Benediktsson H, Burdick JF, Cohen AH, Colvin RB, et al. International standardization of criteria for the histologic diagnosis of renal allograft rejection: the Banff working classification of kidney transplant pathology. Kidney international. 1993;44(2):411-22.
46. Goodwin WE, Kaufman JJ, Mims MM, Turner RD, Glassock R, Goldman R, et al. Human renal transplantation. I, Clinical experiences with six cases of renal homotransplantation. The Journal of

urology. 1963;89(1):13-24.
47. Starzl TE, MARCHIORO TL, WADDELL WR. The reversal of rejection in human renal homografts with subsequent development of homograft tolerance. Surgery, gynecology & obstetrics. 1963;117:385.
48. Feduska NJ, Turcotte JG, Gikas PW, Bacon GE, Penner JA. Reversal of renal allograft rejection with intravenous methylprednisolone "pulse" therapy. Journal of Surgical Research. 1972;12(3):208-15.
49. Gray D, Daar A, Shepherd H, Oliver D, Morris P. Oral versus intravenous high-dose steroid treatment of renal allograft rejection: The big shot or not? The Lancet. 1978;311(8056):117-8.
50. Alarcon-Zurita A, Ladefoged J. Treatment of acute allograft rejection with high doses of corticosteroids. Kidney Int. 1976;9(4):351-4.
51. Rekers NV, Bajema IM, Mallat MJ, Zuidwijk K, Anholts JD, Goemaere N, et al. Quantitative polymerase chain reaction profiling of immunomarkers in rejecting kidney allografts for predicting response to steroid treatment. Transplantation. 2012;94(6):596-602.
52. Rekers N, Bajema I, Mallat M, Anholts J, De Vaal Y, Zandbergen M, et al. Increased metallothionein expression reflects steroid resistance in renal allograft recipients. American Journal of Transplantation. 2013;13(8):2106-18.
53. Monaco AP, Abbott WM, Othersen HB, Simmons RL, Wood ML, Flax MH, et al. Antiserum to lymphocytes: prolonged survival of canine renal allografts. Science. 1966;153(3741):1264-7.
54. STARZL TE, MARCHIORO TL, Porter K, IWASAKI Y, CERILLI GJ. The use of heterologous antilymphoid agents in canine renal and liver homotransplantation and in human renal homotransplantation. Surgery, gynecology & obstetrics. 1967;124(2):301.
55. Mohty M. Mechanisms of action of antithymocyte globulin: T-cell depletion and beyond. Leukemia. 2007;21(7):1387-94.
56. Gaber AO, Monaco AP, Russell JA, Lebranchu Y, Mohty M. Rabbit antithymocyte globulin (Thymoglobulin®). Drugs. 2010;70(6):691-732.
57. Group* OMTS. A randomized clinical trial of OKT3 monoclonal antibody for acute rejection of cadaveric renal transplants. New England Journal of Medicine. 1985;313(6):337-42.
58. Norman DJ. Mechanisms of action and overview of OKT3. Therapeutic drug monitoring. 1995;17(6):615-20.
59. Thistlethwaite JJ, Gaber AO, Haag B, Aronson A, Broelsch C, Stuart J, et al. OKT3 treatment of steroid-resistant renal allograft rejection. Transplantation. 1987;43(2):176-84.
60. Sgro C. Side-effects of a monoclonal antibody, muromonab CD3/orthoclone OKT3: bibliographic review. Toxicology. 1995;105(1):23-9.
61. Baldi A, Malaise J, Mourad M, Squifflet J-P, editors. A prospective randomized study comparing poly-ATG to mono-OKT3 clonal antibodies for the first rejection therapy after kidney transplantation: long-term results. Transplantation proceedings; 2000: Elsevier.
62. Chapman TM, Keating GM. Basiliximab: a review of its use as induction therapy in renal transplantation. Drugs. 2003;63(24):2803-35.
63. Van den Hoogen M, Hesselink D, van Son W, Weimar W, Hilbrands L. Treatment of Steroid-Resistant Acute Renal Allograft Rejection With Alemtuzumab. American Journal of Transplantation. 2013;13(1):192-6.
64. Haynes R, Harden P, Judge P, Blackwell L, Emberson J, Landray MJ, et al. Alemtuzumab-based induction treatment versus basiliximab-based induction treatment in kidney transplantation (the 3C Study): a randomised trial. Lancet (London, England). 2014;384(9955):1684-90.
65. Zheng J, Song W. Alemtuzumab versus antithymocyte globulin induction therapies in kidney transplantation patients: A systematic review and meta-analysis of randomized controlled trials. Medicine. 2017;96(28).
66. Garces JC, Giusti S, Staffeld-Coit C, Bohorquez H, Cohen AJ, Loss GE. Antibody-Mediated Rejection: A Review. The Ochsner journal. 2017;17(1):46-55.
67. Yamada C, Ramon DS, Cascalho M, Sung RS, Leichtman AB, Samaniego M, et al. Efficacy of plasmapheresis on donor-specific antibody reduction by HLA specificity in post–kidney transplant recipients. Transfusion. 2015;55(4):727-35.
68. Slatinska J, Honsova E, Burgelova M, Slavcev A, Viklicky O. Plasmapheresis and Intravenous Immunoglobulin in Early Antibody-Mediated Rejection of the Renal Allograft: A Single-Center Experience. Therapeutic Apheresis and Dialysis. 2009;13(2):108-12.
69. Faguer S, Kamar N, Guilbeaud-Frugier C, Fort M, Modesto A, Mari A, et al. Rituximab therapy for acute humoral rejection after kidney transplantation. Transplantation. 2007;83(9):1277-80.

70. Kaposztas Z, Podder H, Mauiyyedi S, Illoh O, Kerman R, Reyes M, et al. Impact of rituximab therapy for treatment of acute humoral rejection. Clinical transplantation. 2009;23(1):63-73.
71. Lefaucheur C, Nochy D, Andrade J, Verine J, Gautreau C, Charron D, et al. Comparison of Combination Plasmapheresis/IVIg/Anti-CD20 Versus High-Dose IVIg in the Treatment of Antibody-Mediated Rejection. American journal of transplantation. 2009;9(5):1099-107.
72. Locke J, Magro C, Singer A, Segev D, Haas M, Hillel A, et al. The use of antibody to complement protein C5 for salvage treatment of severe antibody-mediated rejection. American Journal of Transplantation. 2009;9(1):231-5.
73. Wongsaroj P, Choi J, Vo A, Kahwaji J, Peng A, Villicana R, et al., editors. Outcomes of Eculizumab (ANTI-C5) Therapy for Treatment of Refractory Antiobdy-Mediated Rejection (ABMR) and Thrombotic Microangiopathy (TMA). AMERICAN JOURNAL OF TRANSPLANTATION; 2015: WILEY-BLACKWELL 111 RIVER ST, HOBOKEN 07030-5774, NJ USA.
74. Kasiske BL, Zeier MG, Chapman JR, Craig JC, Ekberg H, Garvey CA, et al. KDIGO clinical practice guideline for the care of kidney transplant recipients: a summary. Kidney international. 2010;77(4):299-311.
75. Leemans JC, Kors L, Anders H-J, Florquin S. Pattern recognition receptors and the inflammasome in kidney disease. Nature Reviews Nephrology. 2014;10(7):398-414.
76. O'neill LA, Golenbock D, Bowie AG. The history of Toll-like receptors [mdash] redefining innate immunity. Nature Reviews Immunology. 2013;13(6):453-60.
77. Matzinger P. The danger model: a renewed sense of self. Science. 2002;296(5566):301-5.
78. Gluba A, Banach M, Hannam S, Mikhailidis DP, Sakowicz A, Rysz J. The role of Toll-like receptors in renal diseases. Nature reviews Nephrology. 2010;6(4):224-35.
79. Medzhitov R. Toll-like receptors and innate immunity. Nature Reviews Immunology. 2001;1(2):135-45.
80. Kang JY, Nan X, Jin MS, Youn S-J, Ryu YH, Mah S, et al. Recognition of lipopeptide patterns by Toll-like receptor 2-Toll-like receptor 6 heterodimer. Immunity. 2009;31(6):873-84.
81. Jin MS, Kim SE, Heo JY, Lee ME, Kim HM, Paik S-G, et al. Crystal structure of the TLR1-TLR2 heterodimer induced by binding of a tri-acylated lipopeptide. Cell. 2007;130(6):1071-82.
82. Yoon S-i, Kurnasov O, Natarajan V, Hong M, Gudkov AV, Osterman AL, et al. Structural basis of TLR5-flagellin recognition and signaling. Science. 2012;335(6070):859-64.
83. Park BS, Song DH, Kim HM, Choi B-S, Lee H, Lee J-O. The structural basis of lipopolysaccharide recognition by the TLR4–MD-2 complex. nature. 2009;458(7242):1191-5.
84. Tanji H, Ohto U, Shibata T, Miyake K, Shimizu T. Structural reorganization of the Toll-like receptor 8 dimer induced by agonistic ligands. Science. 2013;339(6126):1426-9.
85. Latz E, Verma A, Visintin A, Gong M, Sirois CM, Klein DC, et al. Ligand-induced conformational changes allosterically activate Toll-like receptor 9. Nature immunology. 2007;8(7):772-9.
86. Gay NJ, Symmons MF, Gangloff M, Bryant CE. Assembly and localization of Toll-like receptor signalling complexes. Nature reviews Immunology. 2014;14(8):546-58.
87. Barton GM, Medzhitov R. Toll-like receptor signaling pathways. Science. 2003;300(5625):1524-5.
88. Jenkins KA, Mansell A. TIR-containing adaptors in Toll-like receptor signalling. Cytokine. 2010;49(3):237-44.
89. Kawai T, Akira S. Toll-like Receptor and RIG-1-like Receptor Signaling. Annals of the New York Academy of Sciences. 2008;1143(1):1-20.
90. Kono H, Rock KL. How dying cells alert the immune system to danger. Nature Reviews Immunology. 2008;8(4):279.
91. Leemans JC, Stokman G, Claessen N, Rouschop KM, Teske GJ, Kirschning CJ, et al. Renal-associated TLR2 mediates ischemia/reperfusion injury in the kidney. The Journal of clinical investigation. 2005;115(10):2894-903.
92. Wu H, Chen G, Wyburn KR, Yin J, Bertolino P, Eris JM, et al. TLR4 activation mediates kidney ischemia/reperfusion injury. The Journal of clinical investigation. 2007;117(10):2847-59.
93. Krüger B, Krick S, Dhillon N, Lerner SM, Ames S, Bromberg JS, et al. Donor Toll-like receptor 4 contributes to ischemia and reperfusion injury following human kidney transplantation. Proceedings of the National Academy of Sciences. 2009;106(9):3390-5.
94. Amura CR, Renner B, Lyubchenko T, Faubel S, Simonian PL, Thurman JM. Complement activation and toll-like receptor-2 signaling contribute to cytokine production after renal ischemia/reperfusion. Molecular immunology. 2012;52(3-4):249-57.
95. Andrade-Oliveira V, Campos EF, Goncalves-Primo A, Grenzi PC, Medina-Pestana JO, Tedesco-Silva H, et

al. TLR4 mRNA levels as tools to estimate risk for early posttransplantation kidney graft dysfunction. Transplantation. 2012;94(6):589-95.
96. Stribos EG, van Werkhoven MB, Poppelaars F, van Goor H, Olinga P, van Son WJ, et al. Renal expression of Toll-like receptor 2 and 4: dynamics in human allograft injury and comparison to rodents. Molecular immunology. 2015;64(1):82-9.
97. Janeway Jr CA, Travers P, Walport M, Shlomchik MJ. The complement system and innate immunity. 2001.
98. Damman J, Nijboer WN, Schuurs TA, Leuvenink HG, Morariu AM, Tullius SG, et al. Local renal complement C3 induction by donor brain death is associated with reduced renal allograft function after transplantation. Nephrol Dial Transpl. 2010;26(7):2345-54.
99. Naesens M, Li L, Ying L, Sansanwal P, Sigdel TK, Hsieh S-C, et al. Expression of complement components differs between kidney allografts from living and deceased donors. Journal of the American Society of Nephrology. 2009;20(8):1839-51.
100. Zhou W, Farrar CA, Abe K, Pratt JR, Marsh JE, Wang Y, et al. Predominant role for C5b-9 in renal ischemia/reperfusion injury. The Journal of clinical investigation. 2000;105(10):1363-71.
101. Pratt JR, Basheer SA, Sacks SH. Local synthesis of complement component C3 regulates acute renal transplant rejection. Nature medicine. 2002;8(6):582.
102. Møller-Kristensen M, Wang W, Ruseva M, Thiel S, Nielsen S, Takahashi K, et al. Mannan-Binding Lectin Recognizes Structures on Ischaemic Reperfused Mouse Kidneys and is Implicated in Tissue Injury. Scandinavian journal of immunology. 2005;61(5):426-34.
103. van der Pol P, Schlagwein N, Van Gijlswijk D, Berger S, Roos A, Bajema I, et al. Mannan-Binding Lectin Mediates Renal Ischemia/Reperfusion Injury Independent of Complement Activation. American Journal of Transplantation. 2012;12(4):877-87.
104. Thurman JM, Ljubanovic D, Edelstein CL, Gilkeson GS, Holers VM. Lack of a functional alternative complement pathway ameliorates ischemic acute renal failure in mice. The Journal of Immunology. 2003;170(3):1517-23.
105. Pavlov V, Raedler H, Yuan S, Leisman S, Kwan W-h, Lalli PN, et al. Donor deficiency of decay-accelerating factor accelerates murine T cell-mediated cardiac allograft rejection. The Journal of Immunology. 2008;181(7):4580-9.
106. de Vries B, Kohl J, Leclercq WK, Wolfs TG, van Bijnen AA, Heeringa P, et al. Complement factor C5a mediates renal ischemia-reperfusion injury independent from neutrophils. Journal of immunology. 2003;170(7):3883-9.
107. Peng Q, Li K, Anderson K, Farrar CA, Lu B, Smith RA, et al. Local production and activation of complement up-regulates the allostimulatory function of dendritic cells through C3a–C3aR interaction. Blood. 2008;111(4):2452-61.
108. Peng Q, Li K, Wang N, Li Q, Asgari E, Lu B, et al. Dendritic cell function in allostimulation is modulated by C5aR signaling. The Journal of Immunology. 2009;183(10):6058-68.
109. Li Q, Peng Q, Xing G, Li K, Wang N, Farrar CA, et al. Deficiency of C5aR prolongs renal allograft survival. Journal of the American Society of Nephrology. 2010;21(8):1344-53.
110. Weaver DJ, Reis ES, Pandey MK, Köhl G, Harris N, Gerard C, et al. C5a receptor-deficient dendritic cells promote induction of Treg and Th17 cells. European journal of immunology. 2010;40(3):710-21.
111. Li K, Fazekasova H, Wang N, Peng Q, Sacks SH, Lombardi G, et al. Functional modulation of human monocytes derived DCs by anaphylatoxins C3a and C5a. Immunobiology. 2012;217(1):65-73.
112. Lalli PN, Strainic MG, Yang M, Lin F, Medof ME, Heeger PS. Locally produced C5a binds to T cell–expressed C5aR to enhance effector T-cell expansion by limiting antigen-induced apoptosis. Blood. 2008;112(5):1759-66.
113. Pepys M. Role of complement in induction of antibody production in vivo: effect of cobra factor and other C3-reactive agents on thymus-dependent and thymus-independent antibody responses. Journal of Experimental Medicine. 1974;140(1):126-45.
114. Verschoor A, Brockman MA, Knipe DM, Carroll MC. Cutting edge: myeloid complement C3 enhances the humoral response to peripheral viral infection. The Journal of Immunology. 2001;167(5):2446-51.
115. Marsh JE, Farmer CK, Jurcevic S, Wang Y, Carroll MC, Sacks SH. The Allogeneic T And B Cell Response Is Strongly Dependent On Complement Components C3 And C41. Transplantation. 2001;72(7):1310-8.
116. Havasi A, Borkan SC. Apoptosis and acute kidney injury. Kidney international. 2011;80(1):29-40.
117. Saikumar P, Venkatachalam MA, editors. Role of apoptosis in hypoxic/ischemic damage in the kidney.

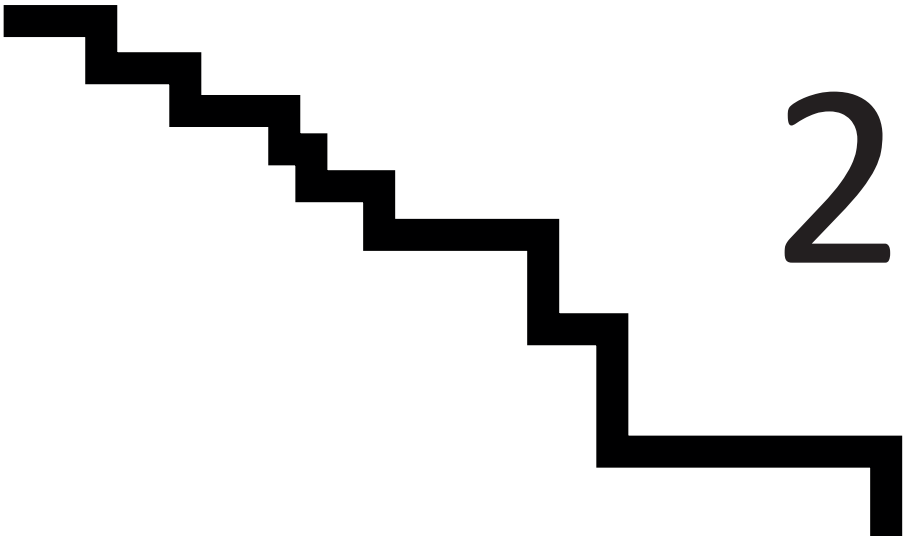
Seminars in nephrology; 2003: Elsevier.
118. Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wideranging implications in tissue kinetics. British journal of cancer. 1972;26(4):239.
119. Dlugosz PJ, Billen LP, Annis MG, Zhu W, Zhang Z, Lin J, et al. Bcl-2 changes conformation to inhibit Bax oligomerization. The EMBO journal. 2006;25(11):2287-96.
120. Youle RJ, Strasser A. The BCL-2 protein family: opposing activities that mediate cell death. Nature reviews Molecular cell biology. 2008;9(1):47.
121. Wolfs TG, De Vries B, Walter SJ, Peutz-Kootstra CJ, Van Heurn L, Oosterhof GO, et al. Apoptotic cell death is initiated during normothermic ischemia in human kidneys. American journal of transplantation. 2005;5(1):68-75.
122. Saikumar P, Dong Z, Mikhailov V, Denton M, Weinberg JM, Venkatachalam MA. Apoptosis: definition, mechanisms, and relevance to disease. The American journal of medicine. 1999;107(5):489-506.
123. Creagh EM. Caspase crosstalk: integration of apoptotic and innate immune signalling pathways. Trends Immunol. 2014;35(12):631-40.
124. Ueda N, Shah SV. Tubular cell damage in acute renal failure—apoptosis, necrosis, or both. Nephrol Dial Transpl. 2000;15(3):318-23.
125. Schumer M, Colombel M, Sawczuk IS, Gobe G, Connor J, O'toole K, et al. Morphologic, biochemical, and molecular evidence of apoptosis during the reperfusion phase after brief periods of renal ischemia. The American journal of pathology. 1992;140(4):831.
126. Miller S, Martin D, Kissane J, editors. Growth-Factors Inhibit The Apoptotic Response To Ischemic Renal Injury In Rats. JOURNAL OF THE AMERICAN SOCIETY OF NEPHROLOGY; 1994: WILLIAMS & WILKINS 351 WEST CAMDEN ST, BALTIMORE, MD 21201-2436.
127. Laine J, Etelämäki P, Holmberg C, Dunkel L. Apoptotic cell death in human chronic renal allograft rejection. Transplantation. 1997;63(1):101-5.
128. Wever P, Aten J, Rentenaar R, Hack C, Koopman G, Weening J. Apoptotic tubular cell death during acute renal allograft rejection. Clinical nephrology. 1998;49(1):28-34.
129. Gregory CD, Devitt A. The macrophage and the apoptotic cell: an innate immune interaction viewed simplistically? Immunology. 2004;113(1):1-14.
130. Ballermann BJ. Glomerular endothelial cell differentiation. Kidney Int. 2005;67(5):1668-71.
131. Özdemir BH, Demirhan B, Özdemir FN, Dalgiç A, Haberal M. The role of microvascular injury on steroid and OKT3 response in renal allograft rejection. Transplantation. 2004;78(5):734-40.
132. Haas M, Kraus ES, Samaniego-Picota M, Racusen LC, Ni W, Eustace JA. Acute renal allograft rejection with intimal arteritis: histologic predictors of response to therapy and graft survival. Kidney international. 2002;61(4):1516-26.
133. Nickeleit V, Vamvakas EC, Pascual M, Poletti BJ, Colvin RB. The prognostic significance of specific arterial lesions in acute renal allograft rejection. Journal of the American Society of Nephrology. 1998;9(7):1301-8.
134. Ho M, Yang E, Matcuk G, Deng D, Sampas N, Tsalenko A, et al. Identification of endothelial cell genes by combined database mining and microarray analysis. Physiological genomics. 2003;13(3):249-62.
135. Sellares J, Reeve J, Loupy A, Mengel M, Sis B, Skene A, et al. Molecular Diagnosis of Antibody-Mediated Rejection in Human Kidney Transplants. American Journal of Transplantation. 2013;13(4):971-83.
136. Reeve J, Sellarés J, Mengel M, Sis B, Skene A, Hidalgo L, et al. Molecular Diagnosis of T Cell-Mediated Rejection in Human Kidney Transplant Biopsies. American Journal of Transplantation. 2013;13(3):645-55.
137. Venner J, Famulski K, Badr D, Hidalgo L, Chang J, Halloran P. Molecular Landscape of T Cell–Mediated Rejection in Human Kidney Transplants: Prominence of CTLA4 and PD Ligands. American Journal of Transplantation. 2014;14(11):2565-76.
138. Halloran PF, Reeve J, Akalin E, Aubert O, Bohmig GA, Brennan D, et al. Real time central assessment of kidney transplant indication biopsies by microarrays: the INTERCOMEX study. American Journal of Transplantation. 2017.
139. Sarwal M, Chua MS, Kambham N, Hsieh SC, Satterwhite T, Masek M, et al. Molecular heterogeneity in acute renal allograft rejection identified by DNA microarray profiling. The New England journal of medicine. 2003;349(2):125-38.
140. Halloran P, Pereira A, Chang J, Matas A, Picton M, De Freitas D, et al. Potential impact of microarray diagnosis of T cell–mediated rejection in kidney transplants: the INTERCOM study. American Journal of

Transplantation. 2013;13(9):2352-63.
141. Rekers NV, Bajema IM, Mallat MJ, Petersen B, Anholts JD, Swings GM, et al. Beneficial Immune Effects of Myeloid-Related Proteins in Kidney Transplant Rejection. American journal of transplantation. 2016;16(5):1441-55.
142. Eikmans M, Roos-van Groningen MC, Sijpkens YW, Ehrchen J, Roth J, Baelde HJ, et al. Expression of surfactant protein-C, S100A8, S100A9, and B cell markers in renal allografts: investigation of the prognostic value. Journal of the American Society of Nephrology. 2005;16(12):3771-86.
143. Waugh N, Cummins E, Royle P, Kandala N, Shyangdan D, Arasaradnam R, et al. Faecal calprotectin testing for differentiating amongst inflammatory and non-inflammatory bowel diseases: systematic review and economic evaluation. 2013.
144. Foell D, Roth J. Proinflammatory S100 proteins in arthritis and autoimmune disease. Arthritis & Rheumatology. 2004;50(12):3762-71.
145. Kane D, Roth J, Frosch M, Vogl T, Bresnihan B, FitzGerald O. Increased perivascular synovial membrane expression of myeloid-related proteins in psoriatic arthritis. Arthritis & Rheumatology. 2003;48(6):1676-85.
146. Frosch M, Strey A, Vogl T, Wulffraat NM, Kuis W, Sunderkötter C, et al. Myeloid-related proteins 8 and 14 are specifically secreted during interaction of phagocytes and activated endothelium and are useful markers for monitoring disease activity in pauciarticular-onset juvenile rheumatoid arthritis. Arthritis & Rheumatology. 2000;43(3):628-37.
147. Odink K, Cerletti N, Brüggen J, Clerc RG, Tarcsay L, Zwadlo G, et al. Two calcium-binding proteins in infiltrate macrophages of rheumatoid arthritis. Nature. 1987;330(6143):80-2.
148. Ryckman C, Vandal K, Rouleau P, Talbot M, Tessier PA. Proinflammatory activities of S100: proteins S100A8, S100A9, and S100A8/A9 induce neutrophil chemotaxis and adhesion. The Journal of Immunology. 2003;170(6):3233-42.
149. Vogl T, Tenbrock K, Ludwig S, Leukert N, Ehrhardt C, Van Zoelen MA, et al. Mrp8 and Mrp14 are endogenous activators of Toll-like receptor 4, promoting lethal, endotoxin-induced shock. Nature medicine. 2007;13(9):1042-9.
150. Boyd JH, Kan B, Roberts H, Wang Y, Walley KR. S100A8 and S100A9 mediate endotoxin-induced cardiomyocyte dysfunction via the receptor for advanced glycation end products. Circulation research. 2008;102(10):1239-46.
151. Leukert N, Vogl T, Strupat K, Reichelt R, Sorg C, Roth J. Calcium-dependent tetramer formation of S100A8 and S100A9 is essential for biological activity. Journal of molecular biology. 2006;359(4):961-72.
152. Murthy A, Lehrer R, Harwig S, Miyasaki K. In vitro candidastatic properties of the human neutrophil calprotectin complex. The Journal of Immunology. 1993;151(11):6291-301.
153. Sohnle PG, Hunter MJ, Hahn B, Chazin WJ. Zinc-reversible antimicrobial activity of recombinant calprotectin (migration inhibitory factor—related proteins 8 and 14). The Journal of infectious diseases. 2000;182(4):1272-5.
154. Cheng P, Corzo CA, Luetteke N, Yu B, Nagaraj S, Bui MM, et al. Inhibition of dendritic cell differentiation and accumulation of myeloid-derived suppressor cells in cancer is regulated by S100A9 protein. Journal of Experimental Medicine. 2008;205(10):2235-49.
155. Sinha P, Okoro C, Foell D, Freeze HH, Ostrand-Rosenberg S, Srikrishna G. Proinflammatory S100 proteins regulate the accumulation of myeloid-derived suppressor cells. The Journal of Immunology. 2008;181(7):4666-75.
156. Zhao F, Hoechst B, Duffy A, Gamrekelashvili J, Fioravanti S, Manns MP, et al. S100A9 a new marker for monocytic human myeloid-derived suppressor cells. Immunology. 2012;136(2):176-83.
157. Luan Y, Mosheir E, Menon M, Wilson D, Woytovich C, Ochando J, et al. Monocytic Myeloid-Derived Suppressor Cells Accumulate in Renal Transplant Patients and Mediate CD4+ Foxp3+ Treg Expansion. American journal of transplantation. 2013;13(12):3123-31.
158. Meng F, Chen S, Guo X, Chen Z, Huang X, Lai Y, et al. Clinical significance of myeloid-derived suppressor cells in human renal transplantation with acute T cell-mediated rejection. Inflammation. 2014;37(5):1799-805.
159. Dessing MC, Tammaro A, Pulskens WP, Teske GJ, Butter LM, Claessen N, et al. The calcium-binding protein complex S100A8/A9 has a crucial role in controlling macrophage-mediated renal repair following ischemia/reperfusion. Kidney international. 2015;87(1):85-94.
160. Nickeleit V, Zeiler M, Gudat F, Thiel G, Mihatsch M. Detection of the complement degradation product

C4d in renal allografts: diagnostic and therapeutic implications. Journal of the American Society of Nephrology. 2002;13(1):242-51.
161. Aiello FB, Furian L, Della Barbera M, Marino S, Seveso M, Cardillo M, et al. Glomerulitis and endothelial cell enlargement in C4d+ and C4d− acute rejections of renal transplant patients. Human pathology. 2012;43(12):2157-66.
162. Vargha R, Mueller T, Arbeiter K, Regele H, Exner M, Csaicsich D, et al. C4d in pediatric renal allograft biopsies: A marker for negative outcome in steroid-resistant rejection. Pediatric transplantation. 2006;10(4):449-53.
163. Botermans JM, de Kort H, Eikmans M, Koop K, Baelde HJ, Mallat MJ, et al. C4d staining in renal allograft biopsies with early acute rejection and subsequent clinical outcome. Clinical Journal of the American Society of Nephrology. 2011;6(5):1207-13.
164. Hippen BE, DeMattos A, Cook WJ, Kew CE, Gaston RS. Association of CD20+ infiltrates with poorer clinical outcomes in acute cellular rejection of renal allografts. American journal of transplantation. 2005;5(9):2248-52.
165. Zarkhin V, Kambham N, Li L, Kwok S, Hsieh S-C, Salvatierra O, et al. Characterization of intra-graft B cells during renal allograft rejection. Kidney international. 2008;74(5):664-73.
166. Kayler LK, Lakkis FG, Morgan C, Basu A, Blisard D, Tan HP, et al. Acute Cellular Rejection with CD20-Positive Lymphoid Clusters in Kidney Transplant Patients Following Lymphocyte Depletion. American Journal of Transplantation. 2007;7(4):949-54.
167. Scheepstra C, Bemelman FJ, van der Loos C, Rowshani AT, Idu MM, ten Berge IJ, et al. B cells in cluster or in a scattered pattern do not correlate with clinical outcome of renal allograft rejection. Transplantation. 2008;86(6):772-8.
168. Jiang Y, Wang R, Wang H, Huang H, Peng W, Qiu W, et al. The Effect of Histological CD20-Positive B Cell Infiltration in Acute Cellular Rejection on Kidney Transplant Allograft Survival. Journal of immunology research. 2016;2016.
169. Özdemir BH, Demirhan B, Güngen Y. The presence and prognostic importance of glomerular macrophage infiltration in renal allografts. Nephron. 2002;90(4):442-6.
170. Özdemir B, Bilezikci B, Haberal A, Demirhan B, Güngen Y, editors. Histologic evaluation, HLA-DR expression, and macrophage density of renal biopsies in OKT3-treated acute rejection: comparison with steroid response in acute rejection. Transplantation proceedings; 2000: Elsevier.
171. Tinckam KJ, Djurdjev O, Magil AB. Glomerular monocytes predict worse outcomes after acute renal allograft rejection independent of C4d status. Kidney international. 2005;68(4):1866-74.
172. Matheson PJ, Dittmer ID, Beaumont BW, Merrilees MJ, Pilmore HL. The macrophage is the predominant inflammatory cell in renal allograft intimal arteritis. Transplantation. 2005;79(12):1658-62.
173. Sun H-j, Zhou T, Wang Y, Fu Y-w, Jiang Y-p, Zhang L-h, et al. Macrophages and T lymphocytes are the predominant cells in intimal arteritis of resected renal allografts undergoing acute rejection. Transplant immunology. 2011;25(1):42-8.
174. Bishop GA, Hall BM, Duggin GG, Horvath JS, Sheil AR, Tiller DJ. Immunopathology of renal allograft rejection analyzed with monoclonal antibodies to mononuclear cell markers. Kidney international. 1986;29(3):708-17.
175. Sarwal MM, Jani A, Chang S, Huie P, Wang Z, Salvatierra Jr O, et al. Granulysin expression is a marker for acute rejection and steroid resistance in human renal transplantation. Human immunology. 2001;62(1):21-31.
176. Desvaux D, Schwarzinger M, Pastural M, Baron C, Abtahi M, Berrehar F, et al. Molecular diagnosis of renal-allograft rejection: correlation with histopathologic evaluation and antirejection-therapy resistance. Transplantation. 2004;78(5):647-53.
177. Muthukumar T, Dadhania D, Ding R, Snopkowski C, Naqvi R, Lee JB, et al. Messenger RNA for FOXP3 in the urine of renal-allograft recipients. New England Journal of Medicine. 2005;353(22):2342-51.
178. Rekers NV, Bajema IM, Mallat MJ, Anholts JD, de Vaal YJ, Zandbergen M, et al. Increased metallothionein expression reflects steroid resistance in renal allograft recipients. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons. 2013;13(8):2106-18.
179. Almoguera B, Shaked A, Keating BJ. Transplantation genetics: current status and prospects. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons. 2014;14(4):764-78.

180. Alakulppi NS, Kyllonen LE, Jantti VT, Matinlauri IH, Partanen J, Salmela KT, et al. Cytokine gene polymorphisms and risks of acute rejection and delayed graft function after kidney transplantation. Transplantation. 2004;78(10):1422-8.
181. Grinyo J, Vanrenterghem Y, Nashan B, Vincenti F, Ekberg H, Lindpaintner K, et al. Association of four DNA polymorphisms with acute rejection after kidney transplantation. Transplant international : official journal of the European Society for Organ Transplantation. 2008;21(9):879-91.
182. Eikmans M, de Canck I, van der Pol P, Baan CC, Haasnoot GW, Mallat MJ, et al. The functional polymorphism Ala258Ser in the innate receptor gene ficolin-2 in the donor predicts improved renal transplant outcome. Transplantation. 2012;94(5):478-85.
183. Brown KM, Kondeatis E, Vaughan RW, Kon SP, Farmer CK, Taylor JD, et al. Influence of donor C3 allotype on late renal-transplantation outcome. The New England journal of medicine. 2006;354(19):2014-23.
184. Varagunam M, Yaqoob MM, Döhler B, Opelz G. C3 polymorphisms and allograft outcome in renal transplantation. New England Journal of Medicine. 2009;360(9):874-80.
185. Gratwohl A, Döhler B, Stern M, Opelz G. HY as a minor histocompatibility antigen in kidney transplantation: a retrospective cohort study. The Lancet. 2008;372(9632):49-53.
186. Kim SJ, Gill JS. HY incompatibility predicts short-term outcomes for kidney transplant recipients. Journal of the American Society of Nephrology. 2009;20(9):2025-33.
187. Zhang X, Reinsmoen NL. Impact of Non-Human Leukocyte Antigen-Specific Antibodies in Kidney and Heart Transplantation. Frontiers in immunology. 2017;8:434.
188. Mesnard L, Muthukumar T, Burbach M, Li C, Shang H, Dadhania D, et al. Exome sequencing and prediction of long-term kidney allograft function. PLoS computational biology. 2016;12(9):e1005088.

The source of SYBR Green master mix determines outcome of nucleic acid amplification reactions



Jianxin Yang, Berit Kemps-Mols, Marijke Spruyt-Gerritse, Jacqueline Anholts, Frans Claas, Michael Eikmans*

Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, the Netherlands.

BMC Res Notes. 2016; 9: 292.

Abstract

Background. Quantitative (q)PCR by amplification of nucleic acid with a fluorescent dye is widely used. Selection of adequate PCR reagents and devices is relevant to achieve reliable and consistent data. Our main objective was to test the robustness of different commercial SYBR green PCR mixes with respect to specificity and sensitivity of the PCR assay, across various PCR machines (Light cycler 96, ViiA7) and amplification protocols. Herein, we applied PCR protocols for determining mRNA transcript levels, DNA copy numbers, and DNA genotype.

Results. First, we set up 70 primer-based assays that targeted immune-related mRNA transcripts. Of the 70 assays 66 (94.3%) resulted in a single melting curve peak, indicating specificity of the amplification, with PCR mixes from large vendors (Roche, ABI, Bio-Rad). But this was only seen when the PCR protocol that was indicated in the vendor's guidelines for each particular mix was applied. When deviating from the prescribed protocol, suboptimal melting curves were most often seen when using Roche SYBR green. With respect to PCR yields, the use of ABI mix more often led to lower Cq values. Second, we set up 20 primer-selective PCR assays to target different insertion-deletion and single nucleotide polymorphism regions throughout the genome. The variation in delta Cq between positive and negative DNA samples among the PCR assays was the lowest when using ABI master mix. Finally, the quality of high resolution melting (HRM) assays for DNA genotyping was compared between four commercial HRM PCR mixes (Roche, Bioline, PCR Biosystems, ABI). Only Roche and ABI mixes produced optimal clusters of melting profiles that clearly distinguished genotype variants.

Conclusion. The current results show a preference for the use of ABI mix when it comes to obtaining higher sensitivity in cDNA analysis and a higher consistency among assays in distinguishing DNA genotypes among different individuals. For HRM assays, it is advisable to use master mix from a relatively large vendor.

Background

Real-time polymerase chain reaction (PCR) is widely used to measure gene expression and DNA copies (1, 2). The most commonly used methods for quantitative polymerase chain reaction (qPCR) are based on non-specific SYBR green chemistry and specific Taqman probe chemistry(3). Intercalating dyes, which bind double–stranded (ds) DNA with high efficiency in the reaction, are most commonly used. When it binds to dsDNA, the fluorescence signal enhances >1,000 fold compared to situation where it is unbound and in free solution (4, 5). The overall fluorescence intensity is proportional to the amplified products and increases as the target is amplified(6). A drawback of SYBR Green I is its lack of specificity: binding to nonspecific dsDNA in the real-time PCR reaction hampers reliable quantification of the specific product(7). Presence of non-specific PCR products can be ruled out by performing a melting curve analysis(8). Therefore, the use of DNA-binding dyes may require more extensive optimization. In general, when performing singleplex assays the use of SYBR green dye is preferable over that of probe chemistry, since the former assays are easier to design, faster to set up, and less expensive (9, 10).

High resolution melting (HRM) analysis is a novel, closed-tube, high-throughput technology for identifying mutations and polymorphisms in nucleic acid sequences(11, 12). The combination of a saturating, DNA-binding dye with superior instrumentation and sophisticated software enables the detection of genetic variations by analyzing PCR melting curves at a finer temperature resolution. HRM reactions generate specific and sensitive melting profiles. They can be used for genotyping, mutation screening, and methylation analysis based on heterozygosity, length, and GC content(13) .

Numerous real-time PCR devices and master mixes are available on the market. To perform reliable high-quality data, PCR master mix, and equipment need to be optimal. However, general lab optimized protocols are widely used for different gene targets and performed diversely between conditions. Our main objective was to test the robustness of different commercial SYBR green PCR mixes with respect to specificity and sensitivity of the PCR assay. This was tested across various PCR machines and amplification protocols for assessment of mRNA transcript levels, DNA copy numbers, and DNA genotypes.

Materials and methods

PCR machines, SYBR Green mixes and HRM mixes

Equipment used included the Light Cycler 96 (Roche Diagnostics, Mannheim, Germany) and the ViiA 7 (Applied Biosystems by Life Technologies, Austin, Texas, USA) real-time PCR machines. Performance of three different PCR mixes was compared, including SYBR Select Master Mix (Applied Biosystems), iQ SYBR Green supermix (Bio-Rad, Hercules, CA, USA), and

FastStart Essential DNA Green Master (Roche Diagnostics). We evaluated four different HRM mixes on the Lighter Cycler 96, namely high resolution melting master (Roche Diagnostics), SensiFast HRM Kit (Bioline, London, UK), qPCRBIO HRM Mix(PCR Biosystems, London, UK), and MeltDoctor HRM Master Mix (Applied Biosystems).

Nucleic acid extraction and cDNA synthesis

DNA was isolated using chemagic DNA Blood2k Kit by chemagic MSM I equipment (PerkinElmer), and the quantity was measured on a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc, Asheville NC). Isolated DNA samples were diluted to 10 ng/µl with nuclease-free water and used as template in qPCR and HRM assays.

RNA was extracted using the NucleoSpin miRNA kit (Macherey-Nagel, Germany) from peripheral blood cells obtained by ficoll or percoll gradients, namely cell subsets positive for either CD3 (T cells) or CD14 (monocytes). Protocols for total RNA purification were followed as described by the manufacturer. RNA quantity was determined on a NanoDrop 2000 Spectrophotometer. RNA quality was evaluated using the StdSense Analysis kit and the Experion RNA analyzer (Bio-Rad, Hercules, CA). Complementary DNA was synthesized from 150 ng of total RNA (RNA quality index>7.0) following the manufacturer's manuals: Superscript III RT (Invitrogen; 200 U of RT), 0.5 mM dNTP, 40 U of RNAse OUT, and 5 mM DTT. RNA was combined with oligo-dT (Invitrogen; 0.25 mg) and random nucleotide hexamers (Invitrogen; 0.25 mg), and incubated at 65°C for 5 minutes(14). The tubes were immediately placed on ice after incubation, and the remaining constituents were added. The reactions were allowed to proceed at 25°C for 5 minutes, at 50°C for 60 minutes, and then terminated at 70°C for 5 minutes.

PCR primers

Optimal primers pairs for cDNA assays were selected using Primer 3 version 4.0.0(15, 16) or Universal Probe Library. To prevent amplification of genomic DNA, forward and reverse primers for majority of the transcripts were designed to target separate exons, spanning at least one intron with a size of 800 bp or more. The PCR efficiency of amplification was calculated by the software using the four-fold serial dilution of pooled cDNA, and 90-110% was considered as acceptable. The primer selection for genomic DNA (gDNA) assays (S01a, S01b, S03, S04a, S04b, S05a, S05b, S06, S07a, S07b, S08a, S08b, S09a, S09b, S10a, S10b, S11a) was based on a previous study (Table 1) (17). Firstly, high percentage of heterozygous biallelic polymorphism in the general population was selected. Second, one of the primer sequences was specific to each allele of polymorphic site, whereas the other one was picked in a common region. HRM primers were designed to amplify a short DNA segment covering polymorphism rs2230199.

Table 1. Primer sequences and amplification efficiency

Target	Forward primer (5'-3')	Reverse primer (5'-3')	Amplicon(bp)	Reagent	Efficiency
GAPDH	acccactcctccacctttgac	tccaccaccctgttgctgtag	110	ABI	0.98
TLR2	gtgataggtgtgaggcaggt	gtggccgccttgattcatag	136	ABI	0.93
CD1c	tttctgcagtttctgctgcta	gagacgtgttcctgggatg	74	ABI	1.06
CD54	ccttcctcaccgtgtactgg	agcgtagggtaaggttcttgc	90	ABI	1.05
CD68	ttcccctatggacacctcag	ttgtactccaccgccatgta	86	ABI	1
CCL4	cctgctgcttttcttacac	cacagacttgcttgcttc	126	ABI	1.09
IL4	gtctcacctcccaactgctt	gttacggtcaactcggtgca	157	Bio Rad	0.99
IL4	gtctcacctcccaactgctt	gttacggtcaactcggtgca	157	Roche	1.01
IL8	gaaggaaccatctcactg	ccactctcaatcactctc	200	Bio Rad	0.96
IL8	gaaggaaccatctcactg	ccactctcaatcactctc	200	Roche	0.94
IL1RN	cctgtcctgtgtcaagtctgg	agcggatgaaggcgaagc	110	ABI	0.93
CEBPB	cgcttacctcggctacca	acgaggaggacgtggagag	65	ABI	0.94
IL-18	tgcatcaactttgtggcaat	atagaggccgatttccttgg	169	ABI	1
V-FOS	actaccactcacccgcagac	ccaggtccgtgcagaagt	75	ABI	0.98
Egr-1	agccctacgagcacctgac	ggtttggctggggtaactg	92	ABI	0.9
Egr-2	ttgaccagatgaacggagtg	tggtttctaggtgcagagacg	121	ABI	0.92
CD43	aagatgtcatcagtgcccca	cacggtgtgggatcctagag	90	ABI	0.93
CCR7	ggtggtggctctccttgtc	actgtggtgttgtctccgatg	84	ABI	1.1
CD40	gcaggcacaaacaagactga	atggcaaacaggatcccgaa	95	ABI	0.91
S01a	ggtaccgggtctccacatga	gggaaagtcactcacccaagg			
S01b	gtaccgggtctccaccagg	gggaaagtcactcacccaagg			
S03	cttttgctttctgtttcttaagggc	tcaatctttgggcaggttgaa			
S04a	ctggtgcccacagttacgct	aaggatgcgtgactgctatgg			
S04b	ctggtgcccacagttacgct	aggatgcgtgactgctcctc			
S05a	aaagtagacacggccagacttagg	catccccacatacggaaaaga			
S05b	agttaaagtagacacggcctccc	catccccacatacggaaaaga			
S06	cagtcaccccgtgaagtcct	tttcccccatctgcctattg			
S07a	tggtattggctttaaaatactggg	tgtacccaaaactcagctgca			
S07b	ggtattggctttaaaatactcaacc	cagctgcaacagttatcaacgtt			
S08a	ctggatgcctcactgatcca	tgggaaggatgcatatgatctg			
S08b	gctggatgcctcactgatgtt	tgggaaggatgcatatgatctg			
S09a	gggcacccgtgtgagtttt	tcagcttgtctgctttctggaa			
S09b	gggcacccgtgtgagtttt	cagcttgtctgctttctgctg			
S10a	gccacaagagactcag	tggcttccttgaggtggaat			
S10b	ttagagccacaagagacaaccag	tggcttccttgaggtggaat			
S11a	taggattcaaccctggaagc	ccagcatgcacctgactaaca			
Hy	ttctggaacctttcttttcaggc	acttccctctgacattacctgataattg			
HA-8p	tgcagtcagcagatcaccc	cttctgggcaacagttatgga			
KIR3-DS1	catcrgttccatgatgcg tccatcggtcccatgatgtt	ccacgatgtccagggga			

qPCR and HRM assays and PCR protocols

The 20-µL qPCR reaction system (cDNA assays) contained 4 µL of 25-times-diluted cDNA, 10 pmol forward and reverse primers, 10 µL of PCR Mix, and nuclease-free water. The 20-µL qPCR reaction (DNA assays) included 50-200 ng DNA, 10 µL of SYBR PCR Mix, 6 pmol forward and reverse primers, and nuclease-free water. The Roche HRM master mix reaction consisted of 7.5 µl of mix, 3 pmol forward and reverse primers, 3 mM MgCl2 , 20 ng DNA, and nuclease-free water. Besides, the 15-µl HRM PCR reaction consisted of 7.5 µl of HRM mix, 6 pmol forward and reverses primers, 20 ng DNA, and nuclease-free water.

The PCR program (cDNA assays) strictly followed the prescribed protocols for each PCR mix (Table 2). Upon completion of each run, a melting curve analysis was performed to check specificity of the primers. In some occasions, the PCR product was additionally analyzed by agarose gel electrophoresis. The quantification cycle (Cq) value represents the number of cycles needed to reach a set threshold fluorescence signal level, which is a measure of number of cDNA or DNA copies.

The HRM PCR program consisted of a pre-incubation for 10 min, followed by 45 cycles of denaturation at 95°C for 10 seconds, annealing at 60°C for 15 seconds, and extension at 72°C for 15 seconds. Melting analysis was performed by first heating to 95°C for 1min, cooling to 40°C for 1min, heating to 65°C, and then melting with continuous acquisition (15 readings/°C) of fluorescence signal until 97°C. Fifteen DNA samples were analyzed, 12 of which were homozygous (GG) and 3 of which were heterozygous (GC) at the SNP location.

Ethics (and consent to participate)

Written informed consent was obtained from donors for use of part of the human material for scientific purposes. Samples were processed and analyzed in an anonymous way. Blood samples used for nucleic acid analysis were obtained in the context of studies performed in accordance with the Declaration of Helsinki Good Clinical Guidelines and approved by the local medical ethics committee.

Data analysis

Statistical analyses were performed using SPSS statistics 20. The mean delta Cq values (positive minus negative gDNA samples) between PCR mixes were compared by paired T test.

Table 2. Prescribed PCR amplification program[1]

Mix	Steps	Temperature	Duration	Cycles
ABI	UDG activation	50°C	2 min	Hold
	Activation	95°C	2 min	Hold
	Denature	95°C	15 sec	40
	Anneal/extend	60°C	60 sec	
	Melt Curve Analysis	95°C	10 sec	
		60°C	60 sec	
		97°C	5 sec	
BioRad	Activation	95°C	3 min	Hold
	Denature	95°C	15 sec	40
	Anneal/extend	60°C	45 sec	
	Melt Curve Analysis	95°C	10 sec	
		55°C	60 sec	
		95°C	15 sec	
Roche	Activation	95°C	10 min	Hold
	Denature	95°C	10 sec	
	Anneal	60°C	10 sec	40
	Extend	72°C	10 sec	
	Melt Curve Analysis	95°C	10 sec	
		65°C	60 sec	
		95°C	15 sec	
General lab PCR program	Activation	95°C	10 min	Hold
	Denature	95°C	15 sec	45
	anneal/Extend	60°C	60 sec	
	Melt Curve Analysis	95°C	10 sec	
		55°C	60 sec	
		97°C	5 sec	

[1]The ramp of each machine were set to default.

Results

Amplification of cDNA

Melting profiles represent a suitable means to distinguish amplified products from primer dimer and other nonspecific amplification artifacts (8, 18). In terms of cDNA templates, 79 immune-related transcripts were targeted by specific primer pairs in PCR reactions containing ABI, Bio Rad or Roche PCR Mix on a Light Cycler 96 PCR device. Of these, 9 primer pairs

showed low performance due to either the absence of amplification product or nonspecific amplification with any of the three different mixes. These were left out of further analysis. The remaining 70 transcripts were classified into four categories according to the melting profiles obtained after PCR with the three different master mixes (Table 3). Sixty-six primer sets (94.3%) generated a single sharp melting peak with all three SYBR green PCR mixes in case of adherence to the suggested PCR protocol in the vendors' guidelines (Table 3, category 1a). In case of using Roche mix in combination with a general lab PCR protocol (Table 2), 13 primer pairs (18.6%) led to suboptimal melting peak after the PCR indicating generation of a specific PCR products (Table 3, category 1b). The primer pair targeting CCL4 showed sharp and specific melting curves only with the ABI and Bio Rad master mix (category 2), while CCL18 showed a single and smooth melting peak only with the Roche mix (category 4). Two primers pairs (those targeting IL8 and IL4; category 3) demonstrated one sharp melting peak with Bio Rad and Roche but negative amplification with ABI mix. Representative melting profiles and gel plots for the categories are shown in figure 1.

Table 3. Categories classified by amplification specificity [1]

Cat	ABI	Bio-Rad	Roche	Transcripts	Number
1a	Y	Y	Y	GAPDH, CD23, CD68, TLR9, Arg1, PDL1, CXCR4, COX2, B-actin, CXCR1, CCL2, CCL3, CD115, CD117, CD11b, CD163, CD14, CD66b, CD86, HLA-DR, IL10, HO-1, IL1b, IL6, S100A9, STAT4, STAT6, STAT3, TGFB1, TNFa, CCL5, CCL7, V-JUN, CSF3R-2, CD13-2, CCR5, CD31, CD44, CD54, CD64, CD16a, CD205, NFkB, S100A8, CCR2, CD62L, MSR1, CCL24, CD15, CD209, CLEC4C, FLT3, IFNγ	66
1b	Y	Y	Y/N [2]	IL-1RN, IL-18, CEBPB, v-FOS, Egr1, Egr2, CD54, CD200R, CD40, CD1c, TLR2, CD43, CCR7	
2	Y	Y	N	CCL4	1
3	Neg	Y	Y	IL8, IL4	2
4	Neg	N	Y	CCL18	1

[1] Y, a single smooth sharp peak; N, more than two or unsmooth peaks; Neg, no amplification
[2] With Roche mix, the primers mentioned generated specific PCR amplicons in the melting curve analysis, only when the suggested PCR protocol from the vendor's guideline (Table 2) was used. In case of using a general lab PCR protocol (Table 2), suboptimal melting curves were observed indicating additional a specific PCR products.

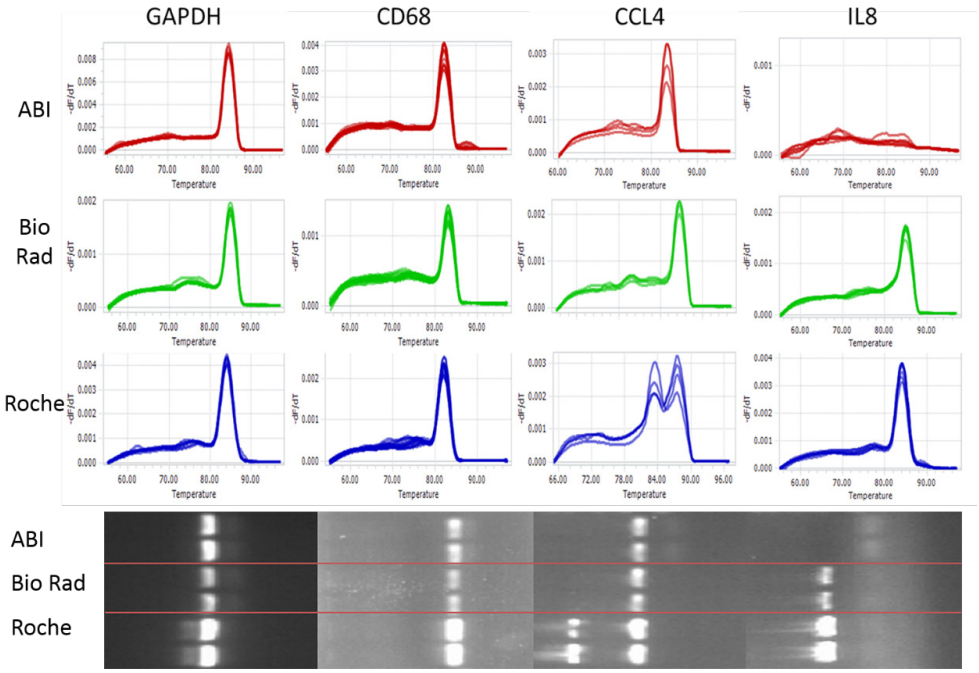


Figure 1. Not all PCR mixes result in optimal specificity of cDNA amplification reactions. The performance of three commercial SYBR green PCR mixes was compared by amplifying cDNA with 70 primer-based assays targeting different mRNA transcripts. A single melting curve peak indicates specificity of the amplification. The figure shows examples of melting curves and corresponding gel blots for several primer sets from Table 3, in situations where all three mixes gave optimal results and where one or more mixes resulted in a suboptimal amplification reaction.

The Cq value is another relevant outcome parameter in quantitative PCR. The difference in Cq value between different PCR mixes was only calculated for the primer sets that gave a specific PCR product with at least two mixes (Figure 2). Delta Cq between PCR mixes varied according to the transcript analyzed and the PCR machine that was used. GAPDH, TLR2, and CD1c showed lower Cq values by Roche mix on a LC96, while lower Cq values were obtained by ABI mix on a ViiA7. Two primer pairs (CD54 and CD68) generated lower Cq values by ABI mix compared with others, which was most prominently observed when using the Viia7 machine. The primer pair of CCL4 produced higher Cq values by ABI mix than the Bio Rad mix on both instruments. Transcript targeting IL8 demonstrated higher Cq values by Roche mix than by Bio Rad mix, whereas IL4 showed lower Cq values by Roche mix on two machines (Figure 2).

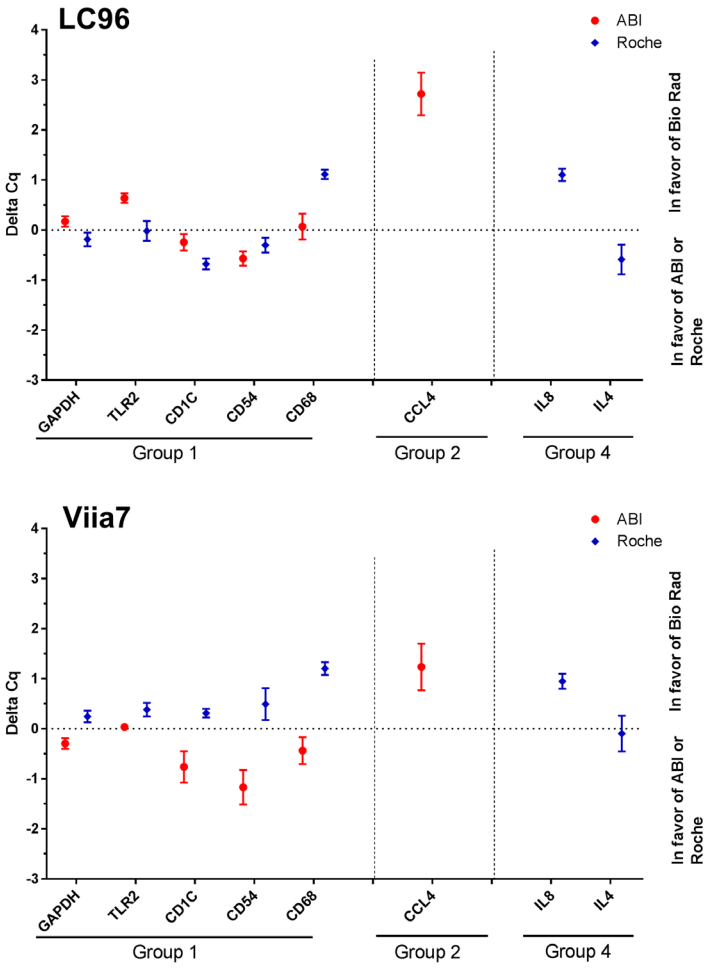


Figure 2. Delta-Cq values between different PCR mixes after cDNA amplification with only those primer pair/PCR mix combinations which led to one specific melting peak. Results for BioRad PCR mix represent the reference (set to zero; black dotted line). Red and blue flags represent results obtained with mixes from ABI and Roche, respectively.

Amplification signals in the no template control (NTC) sample are indicative for primer dimer formation or contamination problems (19). The Bio Rad and Roche mix occasionally showed positive signals with high Cq values (Cq>40) in NTC, while the ABI mix exhibited negative amplification (Cq>45) in most cases (Supplementary figure 1). On minus-reverse-transcriptase controls the ABI mix generated negative amplification (Cq>40) more frequently than the other mixes (Supplementary Figure 1).

Amplification of genomic DNA

Twenty primer-selective PCR SNP assays on genomic DNA were conducted on two different PCR devices. An optimal annealing temperature of 61°C was employed, as tested in a temperature gradient. Absolute Cq values for DNA samples that should be positive or negative for the targeted SNPs are shown in Figure 3A. The mean ΔCq for the 20 assays between positive and negative genomic DNAs was higher with the ABI mix than with the Roche mix (Figure 3b), but this difference was not significant. However, of all mixes tested, the use of ABI mix led to the smallest variation in ΔCq among the different PCR assays (Figure 3B).

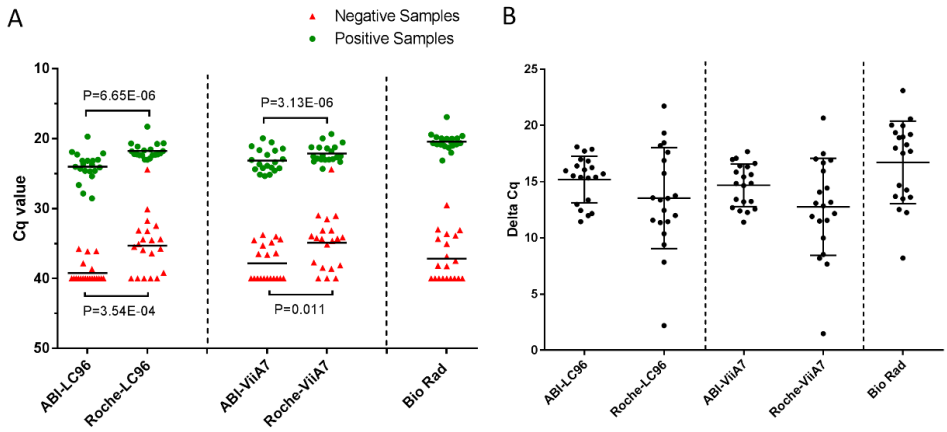


Figure 3. Cq difference between positive and negative genomic DNAs obtained with different PCR mixes and machines. (A) Cq values of 20 primer-selective PCR SNP assays for gDNA samples that should be positive (green dots) or negative (red squares). (B) Individual delta-Cq values for 20 primer-selective PCR SNP assays between positive and negative gDNA samples for ABI and Roche PCR mixes on two different PCR machines. The flags indicate means ± SD.

Genotyping by HRM

For high resolution melting analysis the fluorescent data collected were automatically normalized and derivative melting curve plots were generated (Figure 4). Both the Roche (panel A) and ABI HRM mix (panel D) were able to distinguish the 3 heterozygous samples (GC, orange lines) from the 12 homozygous samples (GG, blue lines). The melt curves from Roche HRM mix were more tightly grouped and easier to separate into clear clusters than ABI HRM mix. With the Bioline HRM mix (panel C) it was also possible to correctly classify the DNA samples according to the right genotype, but the curves were rather unsmooth and tangled. With the PCR Biosystems mix (panel B) none of the three heterozygous DNA samples were correctly classified.

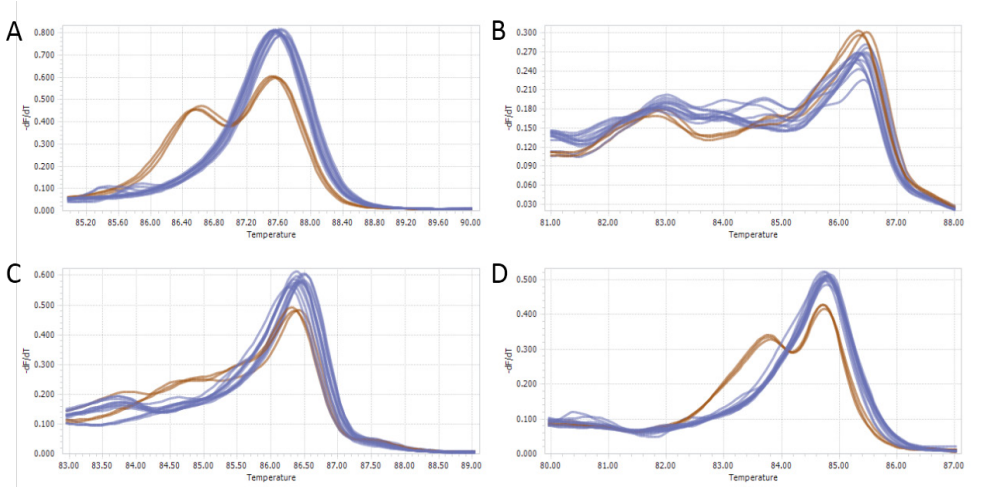


Figure 4. Effect of the type of high resolution melting (HRM) PCR mix on melting curve profiles for distinction of different genotypes. Three DNA samples heterozygous (GC) and 12 DNA samples homozygous (GG) at position rs2230199 were genotyped with HRM using either, (A) high resolution melting master (Roche), (B) qPCRBIO HRM Mix(PCR Biosystems), (C) SensiFast HRM Kit (Bioline), or (D) MeltDoctor HRM Master Mix (Applied Biosystems by Life Technologies). Genotypes were correctly classified with Roche and ABI HRM mixes.

Discussion

Real time PCR technology has been widely accepted because of its high specificity, sensitivity and reproducibility. Selection of appropriate kits is relevant for obtaining reliable results. Here we presented the performance of various SYBR Green PCR mixes and HRM mixes. We wanted to test the robustness of different commercial SYBR green PCR mixes with respect to specificity and sensitivity of the PCR assay.

Sieber and colleagues have shown substantial performance discrepancies among commercial cDNA synthesis kits and qPCR kits in three species (mouse, rat, human) (20); the current study mainly focused on the RT-qPCR process, thereby including specificity of the PCR assays as an essential outcome parameter. Melting curve analysis following PCR amplification can identify the presence of nonspecific amplicons (8, 18). For a subset of primer pairs the melting profile exhibited differences between PCR kits when using one distinct PCR program. However, the poor melting profile markedly improved once the prescribed protocol were strictly followed. This improved amplification may result from the increased extension temperature of the Roche PCR program. Overall, 66 out of 70 transcripts showed a single smooth sharp peak by all commercial PCR kits (Table 3). The transcript targeting CCL4 demonstrated two melting peaks by Roche mix and the PCR products showed

two bands in the gel plot. The primer pairs of IL-8 and IL-4 exhibited negative amplification and absence of PCR products by ABI mix. This discrepancy between transcripts may result from differences in magnesium chloride concentrations between PCR mixes.

When measuring the mRNA expression levels, the PCR amplification efficiency is particularly important(21). The primer sets (GAPDH, TLR2, CD1c, CD54, CD68, CCL4, IL8 and IL4) used for Cq comparison among mixes displayed an acceptable amplification efficiency (Table 1). Two transcripts (CD54, CD68) showed lower Cq values by ABI mix compared to the other mixes on both machines, with even larger disparity on the ViiA7. Interestingly, the CCL4 or IL8 exhibited smaller Cq values by Bio Rad mix than ABI mix or Roche mix, respectively. The inconsistencies in amplification efficiency, especially in categories 2-4, may be due to differences between reagents such as salt concentration and acidity of the solution. Lu showed differences for four genes between ABI and Roche (LC480) PCR systems and also critical effects of magnesium concentration (22). In the current study, we also showed that the ΔCq values between Roche and Bio Rad mix were slightly smaller on the LC96 than on the ViiA7, and similarly, ΔCq values for ABI and Bio Rad mixes were lower on the ViiA7. Therefore, the PCR kit and equipment from the same company are compatible with each other.

DNA chimerism analysis is an useful means to monitor the patient after transplantation, and the PCR assays used for this require high specificity (17, 23). We found that different SYBR green mixes had a different capacity to distinguish positive and negative DNA samples. Although the mean ΔCq between positive and negative DNA samples were not significantly different between PCR mixes, the variation in ΔCq between assays with the ABI mix was smaller than with the Roche and Bio-Rad mixes. This was seen on two different PCR machines. Therefore, we conclude that the ABI PCR mix gives the highest consistency among 20 primer-selective SNP assays on DNA samples.

HRM is a powerful and flexible technique that can be used for genotyping and mutation scanning. The saturating dsDNA-binding dye is one of the important factors for successful HRM analysis. Both Roche and ABI mix could correctly identify the genotype of DNA samples under the identical PCR program conditions (Figure 4). In contrast, the other two HRM mixes generated tangled and unsmooth melting curves, probably because of the quality of PCR amplicon. Our results showed that the source of HRM master mix is a major determinant of successful HRM analysis.

Conclusion

Our data show that three commercial PCR mixes exhibit significant differences with respect to sensitivity of the PCR assay when applying a large panel of primer sets for mRNA transcript quantitation. The consequences of the current findings are that the use of ABI mix has a preference because of higher robustness: this mix more often led to lower Cq values and

a specific PCR reaction, also in case of deviating PCR protocols, compared to other mixes. With primer-selective amplification of genotype variants in genomic DNA samples, ABI PCR mix led to lower background level for negative samples and smaller variation among different assays between positive and negative genomic DNA samples. Overall, the source of the PCR mix had a greater influence on the results than the PCR device used. Finally, with HRM analysis of genomic DNA samples, PCR mixes from Roche and ABI produced the most distinctive melting profiles for correct genotype classification. The present results show that the type of master mix used in nucleic acid amplification reactions determines specificity of the assay and PCR yields.

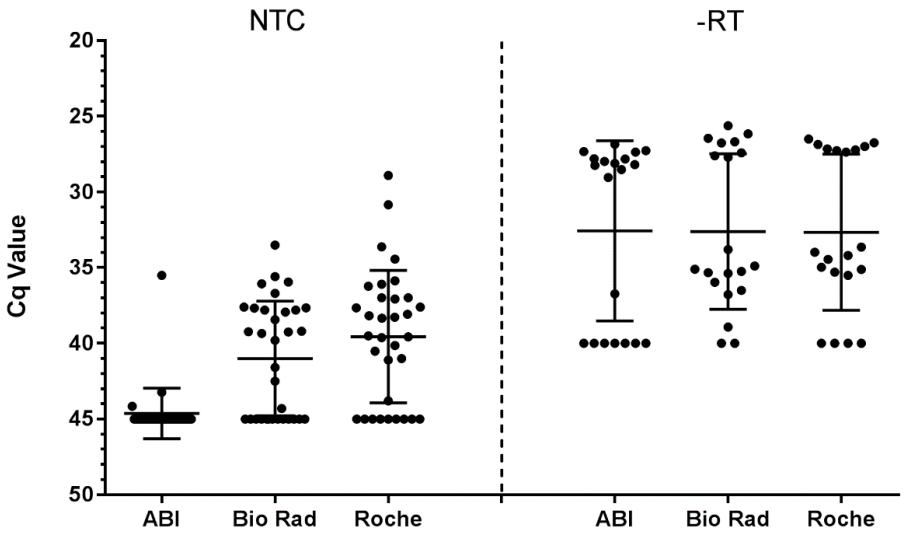
Acknowledgments

J. Yang was awarded financial support from the China Scholarship Council (201306170038).

References

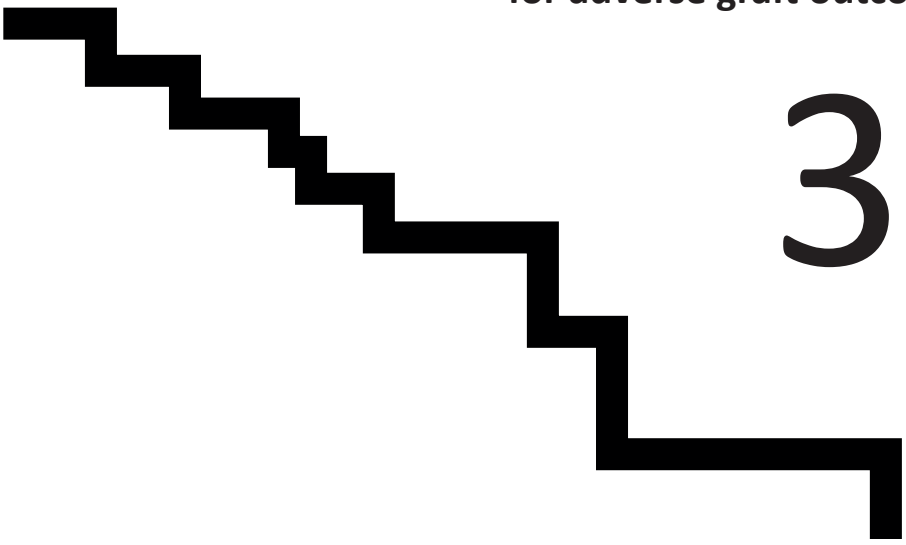
1. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative CT method. Nature protocols. 2008;3(6):1101-8.
2. Ginzinger DG. Gene quantification using real-time quantitative PCR: an emerging technology hits the mainstream. Experimental hematology. 2002;30(6):503-12.
3. Arikawa E, Sun Y, Wang J, Zhou Q, Ning B, Dial SL, et al. Cross-platform comparison of SYBR® Green real-time PCR with TaqMan PCR, microarrays and other gene expression measurement technologies evaluated in the MicroArray Quality Control (MAQC) study. BMC genomics. 2008;9(1):328.
4. Dragan A, Pavlovic R, McGivney J, Casas-Finet J, Bishop E, Strouse R, et al. SYBR Green I: fluorescence properties and interaction with DNA. Journal of fluorescence. 2012;22(4):1189-99.
5. Wittwer CT, Herrmann MG, Moss AA, Rasmussen RP. Continuous fluorescence monitoring of rapid cycle DNA amplification. Biotechniques. 1997;22(1):130-9.
6. Wong ML, Medrano JF. Real-time PCR for mRNA quantitation. Biotechniques. 2005;39(1):75.
7. Ponchel F, Toomes C, Bransfield K, Leong FT, Douglas SH, Field SL, et al. Real-time PCR based on SYBR-Green I fluorescence: an alternative to the TaqMan assay for a relative quantification of gene rearrangements, gene amplifications and micro gene deletions. BMC biotechnology. 2003;3(1):18.
8. Ririe KM, Rasmussen RP, Wittwer CT. Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. Analytical biochemistry. 1997;245(2):154-60.
9. Shu PY, Chang SF, Kuo YC, Yueh YY, Chien LJ, Sue CL, et al. Development of Group- and Serotype-Specific One-Step SYBR Green I-Based Real-Time Reverse Transcription-PCR Assay for Dengue Virus. Journal of Clinical Microbiology. 2003;41(6):2408-16.
10. Yin JL, Shackel NA, Zekry A, McGuinness PH, Richards C, Van Der Putten K, et al. Real-time reverse transcriptase–polymerase chain reaction (RT–PCR) for measurement of cytokine and growth factor mRNA expression with fluorogenic probes or SYBR Green I. Immunology and Cell Biology. 2001;79(3):213-21.
11. Taylor S, Scott R, Kurtz R, Fisher C, Patel V, Bizouam F. A practical guide to High Resolution Melt Analysis Genotyping. Bio-Rad Laboratories. Inc Bulletin. 2010;6004.
12. Gundry CN, Vandersteen JG, Reed GH, Pryor RJ, Chen J, Wittwer CT. Amplicon melting analysis with labeled primers: a closed-tube method for differentiating homozygotes and heterozygotes. Clinical chemistry. 2003;49(3):396-406.
13. Reed GH, Kent JO, Wittwer CT. High-resolution DNA melting analysis for simple and efficient molecular diagnostics. Pharmacogenomics. 2007;8(6):597-608.
14. 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-e9.
15. Untergasser A, Cutcutache I, Koressaar T, Ye J, Faircloth BC, Remm M, et al. Primer3—new capabilities and interfaces. Nucleic acids research. 2012;40(15):e115-e.
16. Koressaar T, Remm M. Enhancements and modifications of primer design program Primer3. Bioinformatics. 2007;23(10):1289-91.
17. Alizadeh M, Bernard M, Danic B, Dauriac C, Birebent B, Lapart C, et al. Quantitative assessment of hematopoietic chimerism after bone marrow transplantation by real-time quantitative polymerase chain reaction. Blood. 2002;99(12):4618-25.
18. Nolan T, Hands RE, Bustin SA. Quantification of mRNA using real-time RT-PCR. Nature protocols. 2006;1(3):1559-82.
19. D'haene B, Vandesompele J, Hellemans J. Accurate and objective copy number profiling using real-time quantitative PCR. Methods. 2010;50(4):262-70.
20. Sieber MW, Recknagel P, Glaser F, Witte OW, Bauer M, Claus RA, et al. Substantial performance discrepancies among commercially available kits for reverse transcription quantitative polymerase chain reaction: a systematic comparative investigator-driven approach. Analytical biochemistry. 2010;401(2):303-11.
21. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, et al. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. Clinical chemistry. 2009;55(4):611-22.
22. Lu S, Smith AP, Moore D, Lee NM. Different real-time PCR systems yield different gene expression values. Molecular and cellular probes. 2010;24(5):315-20.
23. Bai L, Deng YM, Dodds AJ, Milliken S, Moore J, Ma DD. A SYBR green-based real-time PCR method for detection of haemopoietic chimerism in allogeneic haemopoietic stem cell transplant recipients. European journal of haematology. 2006;77(5):425-31.

Supplementary Data



Supplementary Figure 1. Absolute Cq values by three master mixes on no template controls (NTC) and minus reverse transcriptase (-RT) controls.

Elevated intragraft expression of innate immunity and cell death-related markers is a risk factor for adverse graft outcome



3

Jianxin Yang[1], Malou L.H. Snijders[2], Geert W. Haasnoot[1], Cees van Kooten[3], Marko Mallat[3], Johan W. de Fijter[3], Marian C. Clahsen-van Groningen[2], Frans H.J. Claas[1], Michael Eikmans[1]

Depts. of Immunohematology and Blood Transfusion[1] and Nephrology[3]
Leiden University Medical Center, Leiden, the Netherlands.
Dept. of Pathology[2], Erasmus Medical Center,
Rotterdam, the Netherlands

Transplant Immunology. 2018 Jun;48:39-46.

Abstract

Background: Molecules of the innate immune response are increasingly recognized as important mediators in allograft injury during and after kidney transplantation. We therefore aimed to establish the relationship between the expression of these genes at implantation, during an acute rejection (AR) and on graft outcome.
Method: A total of 19 genes, including Toll like receptors (*TLRs*), complement components and regulators, and apoptosis-related genes were analyzed at the mRNA level by qPCR in 123 biopsies with acute rejection and paired pre-transplantation tissue (n=75).
Results: Before transplantation, relative mRNA expression of *BAX:BCL2* ratio (apoptosis marker) and several complement genes was significantly higher in tissue samples from deceased donors compared to living donors. During AR, TLRs and complement genes showed an increased expression compared to pre-transplant conditions, whereas complement regulators were decreased. A relatively high *TLR4* expression level and *BAX:BCL2* ratio during AR in the deceased donor group was associated with adverse graft outcome, independently of clinical risk factors.
Conclusions: Complement- and apoptosis-related gene expression is elevated in deceased donor transplants before transplantation. High *BAX:BCL2* ratio and *TLR4* expression during AR may reflect enhanced intragraft cell death and immunogenic danger signals, and pose a risk factor for adverse graft outcome.

Introduction

The occurrence of an acute kidney allograft rejection, associated with infiltration of recipient immune cells to the kidney, is a risk factor for adverse graft outcome (1). The role of innate immunity including pattern recognition receptors and the complement system in rejection has been appreciated (2, 3). Toll like receptors (TLRs) are a family of transmembrane proteins that are capable of recognizing pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs)(4). TLR stimulation leads to dendritic cell maturation, characterized by upregulation of pro-inflammatory cytokines, chemokines, and co-stimulatory molecules, which initiate an immune response (5). Endogenous ligands including heat-shock proteins (HSP) (6), uric acid (7), high-mobility group box 1 protein (HMGB1) (8, 9), and genomic double-stranded DNA (10) may stimulate TLRs. The interaction between HMGB1 and TLR4 leads to proinflammatory responses in the graft: after kidney transplantation, recipients with a donor graft containing a genotype variant in the coding sequence of TLR4 had lower expression of proinflammatory genes MCP-1 and TNFα and higher expression of anti-inflammatory heme oxygenase 1, and they showed an increased rate of immediate graft function (11). Association of TLR2 and TLR4 expression was found with renal ischemia reperfusion injury (IRI) and early kidney allograft outcomes (12, 13). Other TLRs have not been investigated in the context of delayed graft function (DGF) and acute rejection (AR).

The complement system plays a pivotal role in ischemia reperfusion injury and allograft rejection after transplantation (3). The expression of complement components is significantly increased in deceased donor kidneys after cold ischemia (14, 15). Activation of the complement cascade leads to the release of anaphylatoxins (C3a and C5a) and the formation of the membrane attack complex (MAC) C5b-C9, which mediates the injury following transplantation (16, 17). C2 and C4 are essential components in the classical and lectin pathway, and C3 plays a central role in all pathways of the complement system. Complement regulators act as inhibitors of the complement cascade through various mechanisms (18, 19). For example, the decay acceleration factor (CD55) prevents the formation of C3 convertase. CD46 acts as cofactor for inactivating C3b and C4b by serum factor I. Complement receptor 1 both has decay-accelerating activity and cofactor activity. CD59 prevents the formation of MAC. Deficiency of CD55 and CD59 in experimental settings leads to increased renal ischemia reperfusion injury (20, 21). In C4d-negative biopsy specimens during allograft dysfunction local CD55 expression was related to favorable transplant outcome (22).

The role of apoptosis in IRI after kidney transplantation is increasingly being recognized (23, 24). The anti-apoptotic protein B-cell lymphoma 2 (BCL2) was significantly decreased and pro-apoptotic protein BCL2-associated X protein (BAX) was increased during normothermic ischemia (25). The augmentation of BCL2 protects renal tubular cells from

IRI through reducing renal tubular epithelial cell apoptosis (26). High ratios of *BAX:BCL2* in pre-transplant biopsies are associated with an increased risk of DGF (27).

In the present study, we examined innate-immune-related and apoptosis-related markers in kidney biopsies of 125 patients before transplantation and during an acute rejection episode, and investigated their relation to clinical outcome.

Methods

Patient characteristics

Patients who had received a kidney allograft at the Leiden University Medical Center (LUMC) during 1995-2005 were included. A total of 123 for-cause biopsy samples in case of clinical suspicion of AR were obtained within 6 months after transplantation, and 77 pretransplantation biopsies (75 biopsies paired to the subsequent AR biopsy) were taken at time of transplantation before reperfusion. Patient characteristics are shown in Table 1. Delayed graft function was defined as dialysis-dependency in the first week after transplantation.

Ethics

Written informed consent was obtained from donors for use of part of the human material for scientific purposes. The study were performed in accordance with the Declaration of Helsinki Good Clinical Guidelines and approved by the local medical ethics committee.

Gene selection

The innate immune related genes (*TLR1-TLR10*), potentially acting as initiators of inflammation, were studied. The key complement component (*C2, C3, C4*) and complement regulators (*CR1, CD46, CD55, CD59*), which inhibit complement activation, were included. The apoptosis related genes *BAX* and *BCL2,* which may be associated with IRI and DGF, were also tested.

RNA extraction and cDNA synthesis

RNA isolation and quality check, and cDNA synthesis were performed as described previously (28).

Real time quantitative PCR analysis

Optimal primers pairs were selected using Primer 3 version 4.0.0. To prevent amplification of genomic DNA, reverse and forward primers were designed to target separate exons, spanning at least one intron with a size of 800 bp or more. All primer sets were tested on control cDNA, and PCR efficiencies were between 90% and 110%. The 15-µL qPCR reaction

contained 3 µL of 25-times-diluted cDNA, 15 pmol forward and reverse primers, 7.5 µL of PCR Mix (Applied Biosystems by Life Technologies, Austin, Texas, USA), and nuclease-free water (29). Relative gene expression levels were normalized to the geometric mean of the reference genes β-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Table 1. Demographics of patient cohort.

Variable	Number (%)
Recipient age (>= 50 years)	53 (43.1%)
Recipient gender (Female)	40 (32.5%)
Donor age (>= 50 years)	52 (42.6%)
Donor gender (Female)	74 (60.7%)
Donor type (Living)	24 (19.5%)
Time from transplant to rejection (days, IQR)	14 (9 - 37)
First Transplantation (Yes)	103 (84.4%)
HLA–A/B matching (Yes)	20 (16.4%)
HLA-DR matching (Yes)	43 (35.2%)
Virtual PRA (0-5%)	81 (66.4%)
DGF (Yes)	33 (28.7%)
Steroid responsiveness	68 (56.2%)
Cold ischemia time (<= 18 h)	31 (29.8%)
Banff score	
Glomerulitis (g=0/1/2/3)	74/25/7/3
Interstitial inflammation (i=0/1/2/3)	5/44/36/24
Tubulitis (t=0/1/2/3)	11/39/38/21
Intimal arteritis (v=0/1/2/3)	62/24/7/7
Interstitial fibrosis (ci=0/1/2)	61/41/7
Tubular atrophy (ct=0/1/2)	60/44/5
C4d diffuse positive	14 (11.4%)
Rejection characteristics	
No rejection	7 (5.7%)
Borderline rejection	33 (27.0%)
Interstitial rejection	42 (34.4%)
Vascular rejection	40 (32.8%)
Graft survival (Death censored)	
>1 year	106 (92.2%)
>6 year	101 (87.8%)

HLA, human leukocyte antigen; PRA, panel reactive antibodies; DGF, delayed graft function

Immunohistochemistry

Immunohistochemical studies were performed on an independent set of 34 formalin-fixed and paraffin-embedded (FFPE) kidney biopsy samples: 25 from patients with AR and 9 protocol biopsies from patients with stable graft function. Patients included in this group were transplanted between 2006 and 2015. Monoclonal anti-human antibodies against BAX (ab32503, Abcam, 1:1400 dilution), BCL2 (Sp66, Ventana), TLR4 (ab22048, Abcam, 1:800 dilution), and TLR9 (clone 26C593.2, Novus, 1:800 dilution) were used for immunohistochemistry on sequential 4-µm sections. Staining procedures have been described in a previous publication (30). Semi quantitative scoring of the number of Bcl2-, TLR4-, and TLR9 positive tubular epithelial cells was performed blindly by two observers using a scale from 0 to 5 (0 = 0%, 1 = <10%, 2 = 10-25%, 3 = 26-50%, 4 = 51-75%, 5 = 76-100%).

Statistical analyses

Gene expression differences in paired (PreTx, AR) tissue samples were analyzed using Wilcoxon signed ranks test. Differences in gene expression between deceased and living donors and the occurrence of DGF were assessed by Mann-Whitney U tests (two-sided). Correlations between innate immunity mRNA expression levels and mRNA expression of general inflammation markers were analyzed by Spearman's rank correlation coefficients (two-sided). The Bonferroni method was used to correct for multiple comparisons. Death-censored graft survival curves were created using the Kaplan-Meier method, and differences between curves were calculated using log rank tests. High expression level of inflammatory markers (*CD163, CD68, CD20, CD3e*) was defined as recipients with deceased donor graft with the highest one-third of gene expression. Risk factors affecting graft survival in the deceased donor group were analyzed by multivariate Cox-regression model including the variables showed a borderline significance ($P < 0.1$) in univariate test. Statistical analyses were performed using SPSS statistics, version 23. Due to the limited number of graft loss events, penalized survival analysis by lasso method, including clinical and molecular risk factors, were performed using the "penalized" R (3.4.0 version) package (31).

Results

Relation of pre-transplant gene expression levels with the type of donor

No significant difference was observed between deceased (n=65) and living (n=11) donors regarding the donor age and donor gender. A shorter cold ischemia time (< 18 hours) was more frequently seen in the living-related donation group. Sixteen genes, including the TLRs and membrane-bound complement regulators *C4* and *BAX*, were not significantly different in their expression between living and deceased-related donors at t0 (Table 2). The expression

of the complement genes *C2* and *C3* was more than 4-fold higher in the cadaveric donors compared to the living-related donors (Table 2). A significantly higher *BAX:BCL2* ratio was observed in biopsies of deceased donor kidneys compared to living-related donor kidneys (Figure 1). Within the deceased donation group, recipients with relatively high expression of *C2, C3* and *BAX:BCL2* did not differ from recipients with relatively low expression in the incidence of DGF, steroid resistant rejection, and graft survival (data not shown).

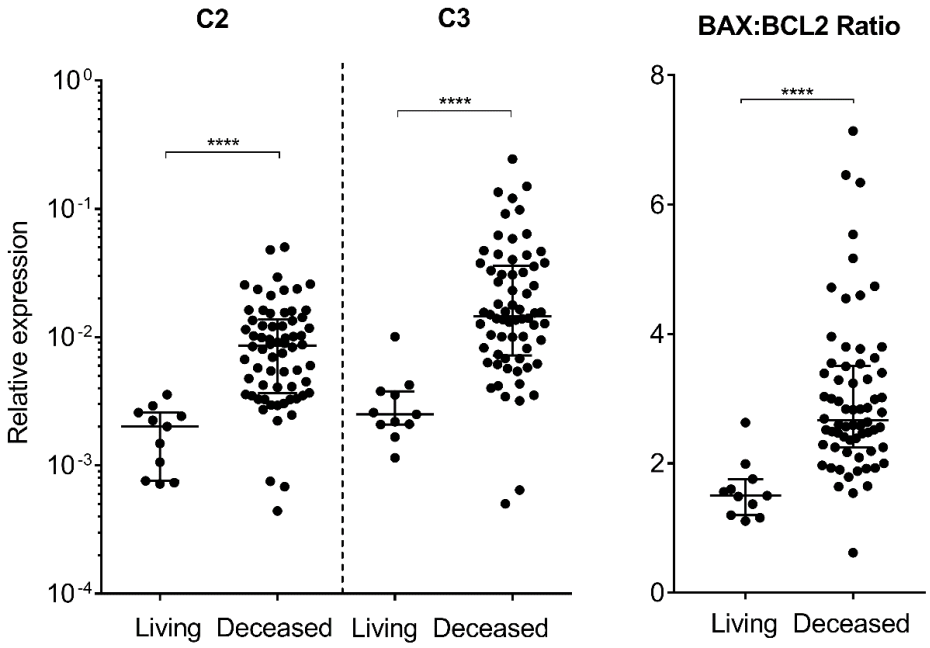


Figure 1. Gene profiling in living and deceased donors at pre-transplant (PreTx). The relative expression of *C2* and *C3* was significantly lower in living donor than that in deceased donor in pre-transplant biopsies. The *BAX:BCL2* ratio was significant lower in living donors in the PreTx biopsies. Flags show median with interquartile range. P values were calculated by Mann-Whitney U tests (two-sided), **** P<0.0001. (corrected for Bonferroni)

No association of gene expression before transplantation with delayed graft function

All recipients with DGF (28.7%) had received a deceased donor renal allograft. Donor age of more than 50 years was a risk factor for DGF. In the pre-implantation tissue of deceased donors, none of the genes investigated were significantly different in expression between patients with DGF and those with no DGF (Table 2).

Table 2. Association between donor type, DGF, and gene expression in the pre-transplant tissues. [a]

	Living (N=11)	Deceased (N=66)	P	DGF (N=22)	No DGF (N=44)	P[f]
TLR1	1.0 (0.86-1.95)	1.45 (0.90-2.06)	0.25	1.05 (0.61-1.56)	1.0 (0.65-1.42)	0.72
TLR2	1.0 (0.38-1.65)	1.42 (0.76-2.38)[b]	0.05	1.10 (0.69-1.87)	1.0 (0.56-1.80)[b]	0.73
TLR3	1.0 (0.79-1.55)	1.28 (0.95-1.66)[b]	0.30	0.99 (0.68-1.55)	1.0 (0.75-1.28)[b]	0.9
TLR4	1.0 (0.69-1.19)	0.96 (0.64-1.34)	0.69	1.25 (0.83-1.65)	1.0 (0.70-1.44)	0.17
TLR5	1.0 (0.82-2.29)	1.20 (0.67-1.89)[b]	0.58	1.45 (0.56-2.05)	1.0 (0.70-1.88)[b]	0.43
TLR6	1.0 (0.48-2.17)	1.26 (0.70-2.49)[b]	0.50	1.14 (0.63-2.29)	1.0 (0.54-2.08)[b]	0.69
TLR7	1.0 (0.64-2.03)	1.68 (1.05-3.07)[c]	0.03	0.86 (0.46-2.05)	1.0 (0.67-1.51)[c]	0.73
TLR8	1.0 (0.62-2.47)	1.49 (0.81-2.45)[e]	0.63	1.53 (1.01-2.76)[b]	1.0 (0.59-2.02)[d]	0.1
TLR9	1.0 (0.82-2.70)	1.00 (0.38-3.13)[b]	0.80	1.19 (0.38-3.68)[b]	1.0 (0.54-3.15)	0.9
TLR10	1.0 (0.44-4.29)	2.14 (0.72-6.42)[c]	0.29	0.90 (0.29-2.54)[b]	1.0 (0.31-3.87)[b]	0.74
CD46	1.0 (0.94-1.15)	0.86 (0.67-1.06)	0.02	0.90 (0.72-1.18)	1.0 (0.77-1.21)	0.45
CD55	1.0 (0.92-1.50)	0.90 (0.63-1.32)	0.09	1.06 (0.84-1.55)	1.0 (0.72-1.53)	0.36
CD59	1.0 (0.83-1.16)	0.97 (0.85-1.26)	0.61	0.99 (0.89-1. 25)	1.0 (0.87-1.29)	0.74
C2	1.0 (0.38-1.28)	4.28 (1.81-6.81)	5.20E-6*	1.01 (0.40-1.72)	1.0 (0.48-1.58)	0.95
C3	1.0 (0.83-1.52)	5.81 (2.88-14.43)	5.98E-6*	1.21 (0.74-2.09)	1.0 (0.47-2.97)	0.59
C4	1.0 (0.89-1.65)	2.17 (1.38-3.03)	0.01	0.97 (0.53-1.39)	1.0 (0.70-1.38)	0.53
CR1	1.0 (0.75-2.07)	0.99 (0.67-1.54)[b]	0.60	1.26 (0.89-1.82)	1.0 (0.68-1.81)[b]	0.41
Bcl2	1.0 (0.87-1.49)	0.71 (0.47-1.01)	2.35E-3*	1.11 (0.66-1.66)	1.0 (0.74-1.37)	0.61
BAX	1.0 (0.83-1.24)	1.18 (0.99-1.53)	0.12	1.13 (1.00-1.58)	1.0 (0.85-1.38)	0.13
BAX:BCL2	1.0 (0.80-1.18)	1.78 (1.50-2.34)	8.41E-5*	1.09 (0.89-1.42)	1.0 (0.87-1.30)	0.34

[a] Gene expression data shown as medians with interquartile range

[b, c, d, e] Data missing for one[b] , two[c] , four[d] , five[e] patients.

[f] The expression level of patients with and without DGF was analyzed in the deceased donor group.

* Statistically significant p-values after Bonferroni correction (P<0.0025). P values were calculated by Mann-Whitney U tests (two-sided).

Comparison of pre-transplant and acute rejection tissues

Paired pre-transplant and acute rejection biopsies of 75 patients were available for analysis of gene expression dynamics (Table 3). The expression level of *TLR 6, TLR7, TLR8, TLR9*, and *TLR10* was elevated more than 5.5 fold at the moment of AR, and the expression levels of *TLR1, TLR2, TLR3*, and *C2* were increased 1.2-4.4 fold compared to those before implantation. The expression levels of *TLR4, TLR5, C3* and *CR1* were similar between both biopsies, and levels of *C4*, *BCL2* and the complement regulators (*CD46, CD55,* and *CD59*) were slightly decreased during AR (Figure 2). Patients whose *C3* expression increased between AR and

pre-transplantation did not differ from patients whose *C3* expression decreased in this time interval with respect to incidence of steroid resistant rejection and death censored graft survival (data not show).

Table 3. Pairwise comparison of gene expression between pre-transplant and AR biopsies.

	Pre-transplant (N=75)	Acute rejection (N=75)	P
TLR1	1.0 (0.65 - 1.46)	4.36 (3.24 - 5.34)	5.50E-14*
TLR2	1.0 (0.52 - 1.63)	3.42 (2.63 - 5.21)	4.60E-12*
TLR3	1.0 (0.74 - 1.29)[a]	1.42 (1.20 - 1.86)[a]	5.38E-8*
TLR4	1.0 (0.70 - 1.37)	1.21 (0.95 - 1.56)	0.019
TLR5	1.0 (0.56 - 1.59)	1.40 (1.01 - 1.70)	0.0028
TLR6	1.0 (0.56 - 1.97)	5.59 (4.18 - 8.47)	2.22E-13*
TLR7	1.0 (0.61 - 1.55)[a]	7.40 (4.60 - 9.79)[a]	1.48E-13*
TLR8	1.0 (0.57 - 1.73)[b]	27.04 (18.94 - 34.87)[b]	3.56E-13*
TLR9	1.0 (0.39 - 3.07)[a]	7.66 (4.99 - 13.14)[a]	1.62E-11*
TLR10	1.0 (0.31 - 2.83)[c]	8.96 (4.55 - 14.31)[c]	2.25E-9*
CD46	1.0 (0.78 - 1.22)	0.80 (0.62 - 1.07)	2.19E-3*
CD55	1.0 (0.71 - 1.48)	0.72 (0.58 - 0.84)	4.34E-7*
CD59	1.0 (0.86 - 1.25)	0.84 (0.63 - 1.04)	4.92E-4*
C2	1.0 (0.47 - 1.76)	2.49 (1.71 - 3.56)	4.34E-7*
C3	1.0 (0.43 - 2.35)	2.04 (1.32 - 3.65)	8.41E-3
C4	1.0 (0.65 - 1.44)	0.49 (0.36 - 0.61)	2.19E-10*
CR1	1.0 (0.70 - 1.54)	1.26 (0.87 - 1.97)	0.0087
BCL2	1.0 (0.67 - 1.35)[c]	0.76 (0.60 – 1.06)[c]	3.14E-4*
BAX	1.0 (0.84 - 1.31)[c]	0.98 (0.85 - 1.27)[c]	0.44
BAX:BCL2	1.0 (0.76 - 1.30)[c]	1.24 (0.95 - 1.49)[c]	6.20E-5*

[a, b, c] Data missing for one[a], five[b], or two[c] patients.

* Statistically significant p-values based on Bonferroni correction (P<0.0025), P values were calculated by Wilcoxon signed ranks test.

Gene expression correlated with inflammatory cell markers and Banff score

Since all TLRs showed elevated levels during AR, we investigated whether this upregulation could be ascribed to infiltration of inflammatory cells. Correlations of innate immunity expression levels with expression of key inflammatory markers (*CD163, CD68, CD20, CD3e*) and Banff classification are summarized in Table S2. Except for *TLR2*, *TLR3* and *TLR5*, all TLRs correlated with one or more inflammatory cell marker. *C2* and *C3* were significantly correlated with macrophage makers, whereas *CD46* and *CD59* showed a negative relationship with these molecules. In addition, *CR1* demonstrated relationships with T cell, B cell, macrophage

markers, and interstitial inflammation score. Apoptosis-related genes did not correlate with any of the inflammatory molecules. In summary, the altered gene expression may in part be the result of infiltrating inflammatory cells.

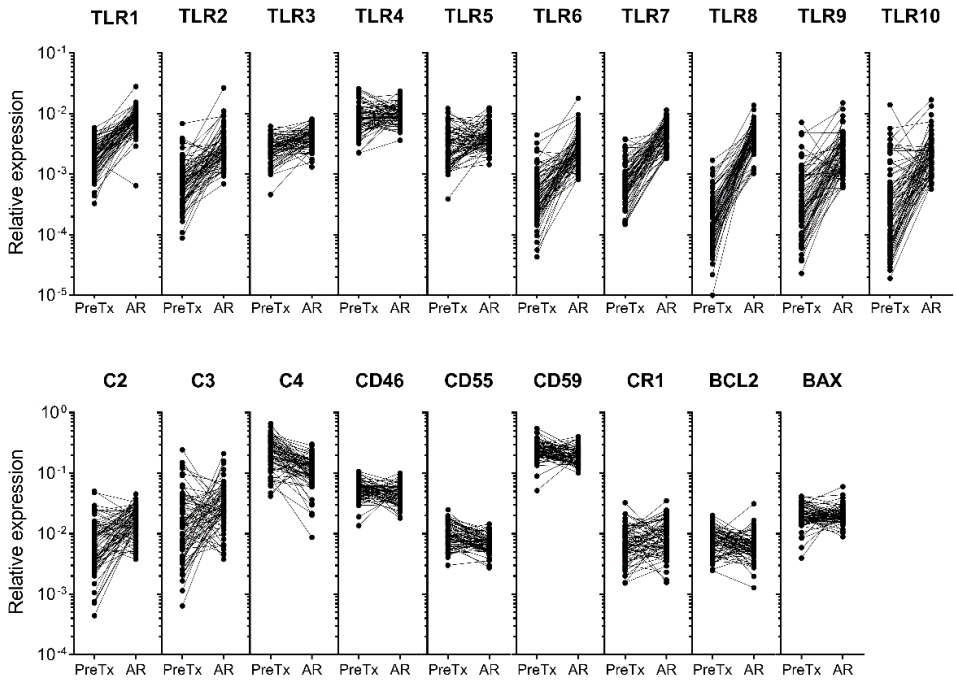


Figure 2. Gene expression dynamics in kidney biopsies. The paired pre-transplant (PreTx) and acute rejection (AR) biopsies of 75 patients were used for comparison. The mRNA levels were quantified by qPCR and normalized to reference genes.

High expression of TLR4 and high BAX:BCL2 ratio during AR predicts inferior graft outcome

The relative expression of TLR4 at the moment of AR in living and deceased patients was comparable. The patients with a deceased donor graft were divided into two groups based on their gene expression levels. One-third of patients who showed the highest *TLR4* expression were defined as high expression group (open circles); and the rest of patients as low expression group (black dots) (Figure 3A). At 12.5 years post transplantation, patients with high *TLR4* expression showed significant inferior graft survival (59.2%) compared to recipients who had relative low *TLR4* expression (79.6%, P= 0.04, Figure 3A). More than 10% of the patients with high *TLR4* expression lost their graft within the first 3 months.

As for the *BAX:BCL2* ratio: patients in the deceased donor group, who had a *BAX:BCL2* ratio that was higher than in the living donation group, were defined as the high ratio group (open circles) (Figure 3B). The group of patients with relatively high *BAX:BCL2* ratio at time of AR had an inferior graft survival (57.9%) compared to patients with a low *BAX:BCL2* ratio (79.8%) and those with a living donor graft (88.3%, P=0.03, Figure 3B). In univariate analysis, Banff classification score did not predict long term graft survival. In multivariate cox regression analysis within deceased donor group (Table 4), only high TLR4 expression (HR=3.46; CI=1.17-10.23; P=0.025) and a high *BAX:BCL2* ratio (HR=4.6; CI=1.44-14.73; P=0.01) were a significant independent risk factor for graft loss. The penalized cox regression model using the lasso showed that high *TLR4* expression, higher donor age (> 50 year) and high *BAX:BCL2* ratio were the most significant (Figure S1). Expression levels in the pre-transplant tissues were not associated with graft survival.

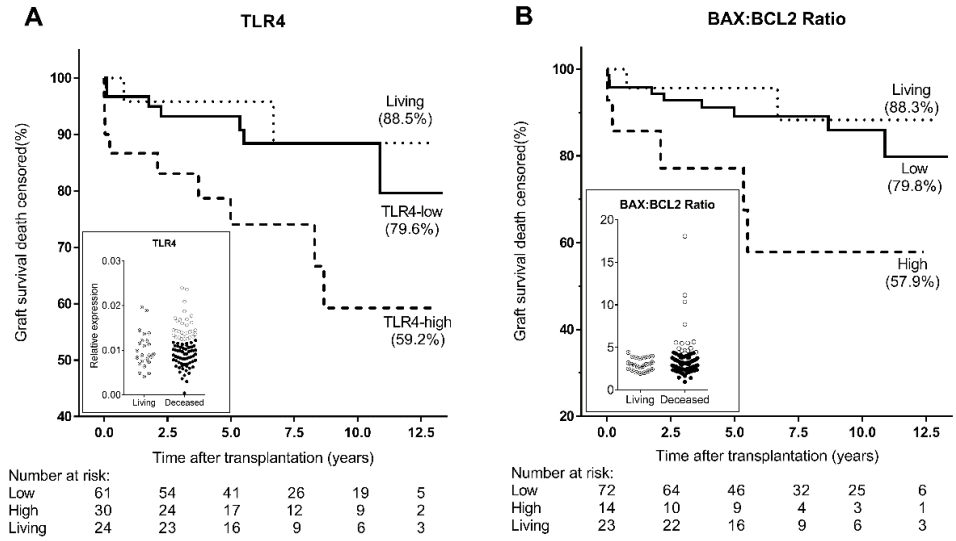


Figure 3. Association between gene expression at moment of acute rejection and kidney graft survival. (A) The *TLR4*-high expression patient group (n=30; dash line) had significantly inferior graft survival compared to the *TLR4*-low expression patient group (n=61; solid line) and living donor group (n=24; dots line). (B) The high *BAX:BCL2* ratio patient group (n=14; dash line) had significantly inferior graft survival rate compared to the low *BAX:BCL2* ratio patient group (n=72; solid line) and the living donor group (n=23; dots line).

Table 4. Cox regression analysis of transplant-related risk factors and post-transplant gene expression levels at time of AR with death censored graft survival.

	Univariate		Multivariate	
	HR (Lower-Upper)	P	HR (Lower-Upper)	P
Recipient age (> 50 year)	1.35 (0.50 - 3.60)	0.55		
Transplantation date (< 1999)	0.93 (0.25 - 3.42)	0.91		
Donor age (> 50 year)	2.10 (0.78 - 5.68)	0.14		
ABDR Mismatching	0.95 (0.27 - 3.34)	0.94		
Cold ischemia time (> 18h)	0.88 (0.25 - 3.14)	0.85		
Delayed Graft function	1.61 (0.60 - 4.33)	0.35		
Vascular rejection	1.28 (0.46 - 3.54)	0.63		
Steroid resistant	1.94 (0.72 - 5.21)	0.19		
Number of transplants (> 1)	2.88 (1.00 - 8.32)	0.05*	–	
CD163 (high expression level)	1.52 (0.55 - 4.20)	0.42		
CD68 (high expression level)	1.78 (0.62 - 5.07)	0.28		
CD20 (high expression level)	0.42 (0.12 - 1.47)	0.17		
CD3e (high expression level)	1.14 (0.41 - 3.16)	0.8		
TLR4 (high expression level)	2.89 (1.08 - 7.78)	0.04*	3.46 (1.17 - 10.23)	0.025*
Ratio *BAX:BCL2* (higher than living)	3.22 (1.09 - 9.51)	0.03*	4.60 (1.44 - 14.73)	0.01*

* Statistically significant difference (P<0.05)

Localization of TLR4, TLR9, and BCL2 expression in renal transplant biopsies

To verify clinically relevant mRNA markers at the protein level and localize their expression in the tissue, immunohistochemical staining for TLR4, BAX and BCL2 were performed on kidney biopsy specimens (Figure 4). In addition, we investigated TLR9 which was increased during AR, and which has been shown to be an inducer of proinflammatory signals (32). Quantification of BAX expression could not be performed since almost no staining was observed in the biopsies (positive area < 10%). TLR4 protein expression was detected in tubular epithelial cells and in inflammatory cells (Figure 4, A and B). Semi-quantitative scoring showed a significantly higher expression during AR than those with stable graft function. Protein expression of TLR9 was predominantly seen in tubular epithelial cells and varied considerably within the AR group (Figure 4, C and D). BCL2 expression was observed in the cytoplasm of tubular epithelial cells and in infiltrating inflammatory cells, and showed a wide range of expression among AR biopsy samples (Figure 4, E and F). The extent of protein expression of BCL2 and TLR9 during AR was increased in comparison to the stable graft group, however this difference was not significant after correction for multiple comparisons (Table 5).

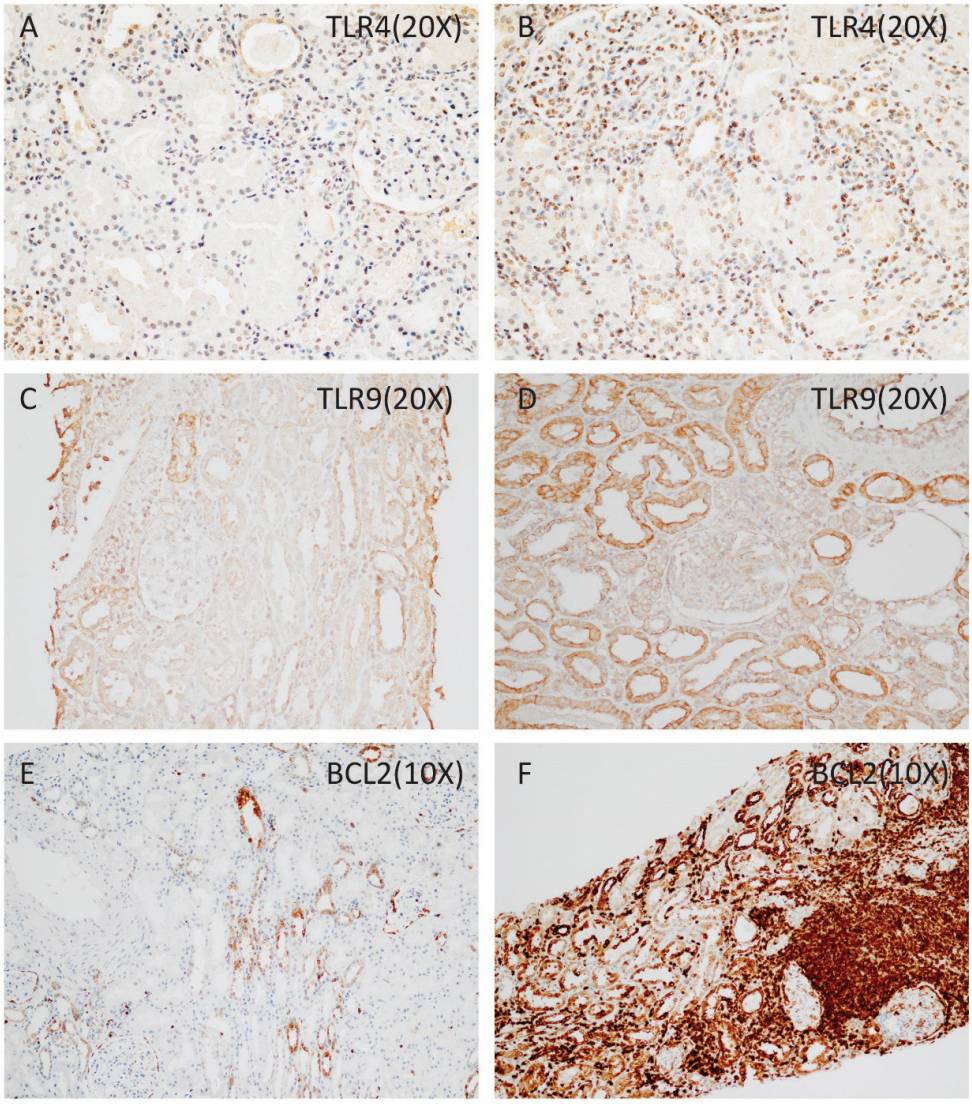


Figure 4. Immunohistochemical staining pattern of TLR4, TLR9, and BCL2 in kidney transplant biopsy specimens. TLR4 protein expression was detected in tubular epithelial cells and in inflammatory cells (A-B). TLR9 was observed in the tubular epithelial cells (C-D). BCL2 was detected in tubular epithelial cells and infiltrated lymphocytes (E-F). Both BCL2 and TLR9 expression varied extensively between acute rejection biopsy specimens. Two representative samples from the acute rejection group are shown.

Table 5. Immunohistochemical scoring of TLR4, TLR9, and BCL2 in stable graft function and acute rejection biopsies.

IHC score	1	2	3	4	5	P
TLR9						0.069
SGF	1	5	1	0	0	
AR	4	4	7	4	4	
TLR4						0.008*
SGF	4	2	1	0	0	
AR	2	7	5	5	3	
BCL2						0.024
SGF	1	3	2	1	0	
AR	0	3	8	8	3	

IHC, Immunohistochemistry; SGF, stable graft function; AR, acute rejection.
* Statistically significant p-values based on Bonferroni correction (P<0.016), P values were calculated by Mann-Whitney Test.

Discussion

In the present study mRNA expression levels of TLRs, key complement components and regulators, and apoptosis-related genes were investigated in biopsies obtained before graft implantation and at time of AR. We found that in deceased donors, *C2* and *C3* expression and *BAX:BCL2* ratio are already elevated before transplantation but were not indicative of DGF. High *TLR4* levels and a high *BAX:BCL2* ratio at the time of an AR were both independent risk factors of graft loss. Results from this exploratory study suggest that innate immune activation occurs both at time of graft implantation and during episodes of acute rejection.

Although the TLR/MyD88 pathway was found to be redundant for host defense against most natural infections (33), depletion of a functional TLR pathway in mice, by knocking out either TLR2, TLR4 or MyD88, protects against IRI and kidney dysfunction, and limits an increase in expression of cytokines, chemokines and in infiltration of inflammatory cells (34, 35). In human kidney transplants, the expression of TLR4 and HMGB1 (an endogenous ligand of TLR4) was significantly elevated in pre-implementation biopsies from deceased donors in contrast to those from living donors (11). However, in our study, we could not confirm these findings (Table 2), and none of the markers we investigated were associated with DGF.

Earlier studies showed that the expression of TLRs is significantly upregulated during allograft rejection mainly because of infiltration of leukocytes (13, 36). However, none of the previous studies have documented gene expression dynamics in a large patient cohort. We

showed that the expression of all TLRs except *TLR4* and *TLR5* was significantly increased in AR biopsies when compared to pre-implementation biopsies (Table 3). Moreover, expression of majority of the TLRs positively correlated with one or more inflammatory cell markers at the moment of AR (Table S1), suggesting that the elevated expression of TLRs is a result of inflammatory cell presence. As for *TLR2* and *TLR3*, which showed only minor increase during an AR but they were not correlated with any inflammatory markers, their expression may be dominant in renal parenchymal tissue. Similar expression patterns of *TLR3* were reported by Dessing et al. (36). *TLR4*, the expression of which correlated with *CD163* and *CD68* but was not increased during AR, may be expressed by both parenchymal and myeloid cells. In addition, patients with relatively high levels of TLR4 during AR exhibited inferior graft survival 12.5 years after transplantation, which may mean that intracellular ligands released after cell damage bind to TLR4 and thereby provide additional inflammatory signals leading to long term graft loss. Expression of TLR4 in the renal allograft biopsy has been described previously (11, 13). TLR4 was expressed in tubular cells and infiltrated lymphocytes, with significantly higher expression during AR compared to stable graft conditions. The possible explanation, that on one hand no increase in *TLR4* mRNA was seen between AR and pre-Tx and on the other hand immunohistochemistry showed significantly higher expression during AR compared to stable graft conditions, may be that the epithelium expresses high levels of mRNA but relatively low level of protein. The endogenous pattern recognition receptor TLR9 is involved in immune complex kidney disease (37). Immunohistochemical staining showed that TLR9 was increased during AR compared to the stable graft group with borderline significance.

The complement system acts as a bridge to the adaptive system and facilitates clearance of immune complexes and cellular debris. It has been shown that the MAC plays a central role in renal IRI and that locally synthesized C3 is important in kidney graft survival (17, 38). In line with a previous study (14), the mRNA levels of *C2* and *C3* in the living donor grafts were significantly lower than those in the deceased donor grafts at time of implantation, which supports the notion that the local C3 expression is induced by donor brain death (15). The observations of a slight increase in C4 expression in deceased donors are in line with those from a previous study (14). However, inconsistent with that study, the expression level of CR1 was comparable between deceased and living donor biopsies in our relatively large cohort. The increased C2 during AR may represent a higher activity of the classical and lectin pathway, whereas the decreased C4 expression may be a result of injury of renal parenchymal cells.

The complement regulators CD46, CD55, and CD59 act as inhibitors of activation of the complement pathway. Hyper-sensitized rats treated with sCR1 displayed significantly prolonged cardiac graft survival (39). Similarly, kidneys of animals treated with CR1 derivatives (APT070) showed less acute tubular injury, and the animals had a significantly higher graft survival rate (40). CD55 had a protective effect on renal function in C4d-negative

grafts and antibody-mediated cardiac allograft rejection (22, 41). We found that expression of *CD46, CD55* and *CD59* was significantly reduced during an AR compared to that in the pre-transplantation tissue. However, none of the complement regulators were predictive for the development of DGF, steroid resistant rejection and graft survival in the present study. Interestingly, Budding et al. showed that serum sCD59 are elevated at the time of bronchiolitis obliterans syndrome (BOS) after lung transplantation, and the patients with higher serum sCD59 titers (>400pg/ml) had a significantly lower chance of BOS free survival. We observed that the expression of complement regulators at time of AR was slightly decreased compared to pre-transplant conditions, and that it negatively correlated with expression of macrophage markers.

It has been shown that kidney cell apoptosis is involved in IRI and that apoptotic cells are frequently present in AR biopsies (23, 24). In the present study, the mRNA of *BCL2*, an anti-apoptotic molecule, was lower in deceased donor biopsies than in living donor grafts. The higher *BAX:BCL2* ratio suggests that the extent of apoptosis is already increased in deceased donors. The *BAX:BCL2* ratio tended to be higher in the DGF group in the deceased donor cohort, but this was only marginal (Table 2), which is inconsistent with findings from a previous study (27). During an AR, the *BAX:BCL2* ratio was marginally increased and it significantly correlated with expression of macrophage markers. Protein investigations by immunohistochemical staining showed that BAX was rarely detected in the biopsy samples. The BCL2 expression was mainly observed in tubular epithelial cell and inflammatory cells, with a wide range of staining within the AR group. Patients who received a living donor graft had superior graft survival compared to those with a graft from a deceased donor, and thus this group acted as a reference. Moreover, patients with relatively high *BAX:BCL2* ratio during AR in their deceased donor graft demonstrated significantly inferior graft survival rates (57.9%) 12.5 years after transplantation compared to those with a lower ratio or to patients who had received a living donor graft (Figure 3). High *BAX:BCL2* ratio during AR possibly reflects an increased number of apoptotic cells, which leads to attraction of phagocytic cells to the graft (42, 43). The accumulated phagocytes may be triggered by immunogenic danger signals and mediate subsequent chronic allograft loss (44, 45).

In conclusion, complement and apoptosis pathways are elevated before kidney transplantation. Increased expression of the majority of genes partly reflect the infiltration of inflammatory cells during an AR. Relatively high *TLR4* expression and *BAX:BCL2* ratio during AR, possibly reflecting enhanced immunogenic danger signals, were both independent risk factors for adverse outcome after transplantation of a deceased donor kidney. The results of this study suggest that the different impact of AR on outcome between living and deceased donor transplants may partly be ascribed to differences in TLR4 regulation and cell death related mechanisms. They form a basis to further validate and explore the functional relevance of these pathways in relation to transplant outcome.

Reference

1. Suthanthiran M, Strom TB. Renal transplantation. The New England journal of medicine. 1994;331(6):365-76.
2. Wood KJ, Goto R. Mechanisms of rejection: current perspectives. Transplantation. 2012;93(1):1-10.
3. Sacks SH, Zhou W. The role of complement in the early immune response to transplantation. Nature Reviews Immunology. 2012;12(6):431-42.
4. Janeway Jr CA, Medzhitov R. Innate immune recognition. Annual review of immunology. 2002;20(1):197-216.
5. Matzinger P. The danger model: a renewed sense of self. Science. 2002;296(5566):301-5.
6. Ohashi K, Burkart V, Flohe S, Kolb H. Cutting edge: heat shock protein 60 is a putative endogenous ligand of the toll-like receptor-4 complex. The Journal of Immunology. 2000;164(2):558-61.
7. Shi Y, Evans JE, Rock KL. Molecular identification of a danger signal that alerts the immune system to dying cells. Nature. 2003;425(6957):516-21.
8. Scaffidi P, Misteli T, Bianchi ME. Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. Nature. 2002;418(6894):191-5.
9. Park JS, Svetkauskaite D, He Q, Kim JY, Strassheim D, Ishizaka A, et al. Involvement of toll-like receptors 2 and 4 in cellular activation by high mobility group box 1 protein. Journal of Biological Chemistry. 2004;279(9):7370-7.
10. Ishii KJ, Suzuki K, Coban C, Takeshita F, Itoh Y, Matoba H, et al. Genomic DNA released by dying cells induces the maturation of APCs. Journal of immunology. 2001;167(5):2602-7.
11. Kruger B, Krick S, Dhillon N, Lerner SM, Ames S, Bromberg JS, et al. Donor Toll-like receptor 4 contributes to ischemia and reperfusion injury following human kidney transplantation. Proceedings of the National Academy of Sciences. 2009;106(9):3390-5.
12. Andrade-Oliveira V, Campos EF, Goncalves-Primo A, Grenzi PC, Medina-Pestana JO, Tedesco-Silva H, et al. TLR4 mRNA levels as tools to estimate risk for early posttransplantation kidney graft dysfunction. Transplantation. 2012;94(6):589-95.
13. Stribos EG, van Werkhoven MB, Poppelaars F, van Goor H, Olinga P, van Son WJ, et al. Renal expression of Toll-like receptor 2 and 4: dynamics in human allograft injury and comparison to rodents. Molecular immunology. 2015;64(1):82-9.
14. Naesens M, Li L, Ying L, Sansanwal P, Sigdel TK, Hsieh SC, et al. Expression of complement components differs between kidney allografts from living and deceased donors. Journal of the American Society of Nephrology. 2009;20(8):1839-51.
15. Damman J, Nijboer WN, Schuurs TA, Leuvenink HG, Morariu AM, Tullius SG, et al. Local renal complement C3 induction by donor brain death is associated with reduced renal allograft function after transplantation. Nephrol Dial Transpl. 2010;26(7):2345-54.
16. Damman J, Schuurs TA, Ploeg RJ, Seelen MA. Complement and renal transplantation: from donor to recipient. Transplantation. 2008;85(7):923-7.
17. Zhou W, Farrar CA, Abe K, Pratt JR, Marsh JE, Wang Y, et al. Predominant role for C5b-9 in renal ischemia/reperfusion injury. Journal of Clinical Investigation. 2000;105(10):1363.
18. Roumenina LT, Zuber J, Fremeaux-Bacchi V. Physiological and therapeutic complement regulators in kidney transplantation. Current opinion in organ transplantation. 2013;18(4):421-9.
19. Zipfel PF, Skerka C. Complement regulators and inhibitory proteins. Nature Reviews Immunology. 2009;9(10):729-40.
20. Yamada K, Miwa T, Liu J, Nangaku M, Song WC. Critical protection from renal ischemia reperfusion injury by CD55 and CD59. The Journal of Immunology. 2004;172(6):3869-75.
21. Turnberg D, Botto M, Lewis M, Zhou W, Sacks SH, Morgan BP, et al. CD59a deficiency exacerbates ischemia-reperfusion injury in mice. The American journal of pathology. 2004;165(3):825-32.
22. Brodsky SV, Nadasdy GM, Pelletier R, Satoskar A, Birmingham DJ, Hadley GA, et al. Expression of the decay-accelerating factor (CD55) in renal transplants - a possible prediction marker of allograft survival. Transplantation. 2009;88(4):457-64.
23. Havasi A, Borkan SC. Apoptosis and acute kidney injury. Kidney Int. 2011;80(1):29-40.
24. Kosieradzki M, Rowinski W, editors. Ischemia/reperfusion injury in kidney transplantation: mechanisms and prevention: Elsevier; 2008.
25. Wolfs TGAM, de Vries B, Walter SJ, Peutz-Kootstra CJ, van Heurn LWE, Oosterhof GON, et al.

Apoptotic cell death is initiated during normothermic ischemia in human kidneys. American Journal of Transplantation. 2005;5(1):68-75.
26. Suzuki C, Isaka Y, Shimizu S, Tsujimoto Y, Takabatake Y, Ito T, et al. Bcl-2 protects tubular epithelial cells from ischemia reperfusion injury by inhibiting apoptosis. Cell transplantation. 2008;17(1-2):223-9.
27. Goncalves-Primo A, Mourao TB, Andrade-Oliveira V, Campos EF, Medina-Pestana JO, Tedesco-Silva H, et al. Investigation of apoptosis-related gene expression levels in preimplantation biopsies as predictors of delayed kidney graft function. Transplantation. 2014;97(12):1260-5.
28. Rekers NV, Bajema IM, Mallat MJ, Zuidwijk K, Anholts JD, Goemaere N, et al. Quantitative polymerase chain reaction profiling of immunomarkers in rejecting kidney allografts for predicting response to steroid treatment. Transplantation. 2012;94(6):596-602.
29. Yang J, Kemps-Mols B, Spruyt-Gerritse M, Anholts J, Claas F, Eikmans M. The source of SYBR green master mix determines outcome of nucleic acid amplification reactions. BMC research notes. 2016;9(1):1.
30. Schonkeren D, van der Hoorn M-L, Khedoe P, Swings G, van Beelen E, Claas F, et al. Differential distribution and phenotype of decidual macrophages in preeclamptic versus control pregnancies. The American journal of pathology. 2011;178(2):709-17.
31. Goeman JJ. L1 penalized estimation in the Cox proportional hazards model. Biometrical journal. 2010;52(1):70-84.
32. Chen L, Ahmed E, Wang T, Wang Y, Ochando J, Chong AS, et al. TLR signals promote IL-6/IL-17-dependent transplant rejection. The Journal of Immunology. 2009;182(10):6217-25.
33. Von Bernuth H, Picard C, Jin Z, Pankla R, Xiao H, Ku C-L, et al. Pyogenic bacterial infections in humans with MyD88 deficiency. Science. 2008;321(5889):691-6.
34. Leemans JC, Stokman G, Claessen N, Rouschop KM, Teske GJ, Kirschning CJ, et al. Renal-associated TLR2 mediates ischemia/reperfusion injury in the kidney. The Journal of clinical investigation. 2005;115(10):2894-903.
35. Wu H, Chen G, Wyburn KR, Yin J, Bertolino P, Eris JM, et al. TLR4 activation mediates kidney ischemia/reperfusion injury. The Journal of clinical investigation. 2007;117(10):2847-59.
36. Dessing MC, Bemelman FJ, Claessen N, Ten Berge IJ, Florquin S, Leemans JC. Intragraft Toll-like receptor profiling in acute renal allograft rejection. Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association. 2010;25(12):4087-92.
37. Anders H-J, Banas B, Schlöndorff D. Signaling danger: toll-like receptors and their potential roles in kidney disease. Journal of the American Society of Nephrology. 2004;15(4):854-67.
38. Pratt JR, Basheer SA, Sacks SH. Local synthesis of complement component C3 regulates acute renal transplant rejection. Nature medicine. 2002;8(6):582-7.
39. Pruitt SK, Bollinger RR. The effect of soluble complement receptor type 1 on hyperacute allograft rejection. Journal of Surgical Research. 1991;50(4):350-5.
40. Patel H, Smith RA, Sacks SH, Zhou W. Therapeutic strategy with a membrane-localizing complement regulator to increase the number of usable donor organs after prolonged cold storage. Journal of the American Society of Nephrology. 2006;17(4):1102-11.
41. Gonzalez-Stawinski GV, Tan CD, Smedira NG, Starling RC, Rodriguez ER. Decay-accelerating factor expression may provide immunoprotection against antibody-mediated cardiac allograft rejection. J Heart Lung Transpl. 2008;27(4):357-61.
42. Lauber K, Bohn E, Kröber SM, Xiao Y-j, Blumenthal SG, Lindemann RK, et al. Apoptotic cells induce migration of phagocytes via caspase-3-mediated release of a lipid attraction signal. Cell. 2003;113(6):717-30.
43. Gregory CD, Devitt A. The macrophage and the apoptotic cell: an innate immune interaction viewed simplistically? Immunology. 2004;113(1):1-14.
44. Ricardo SD, Van Goor H, Eddy AA. Macrophage diversity in renal injury and repair. The Journal of clinical investigation. 2008;118(11):3522-30.
45. Rekers NV, Bajema IM, Mallat MJ, Petersen B, Anholts JD, Swings GM, et al. Beneficial Immune Effects of Myeloid-Related Proteins in Kidney Transplant Rejection. American journal of transplantation. 2016;16(5):1441-55.

Supplementary Data

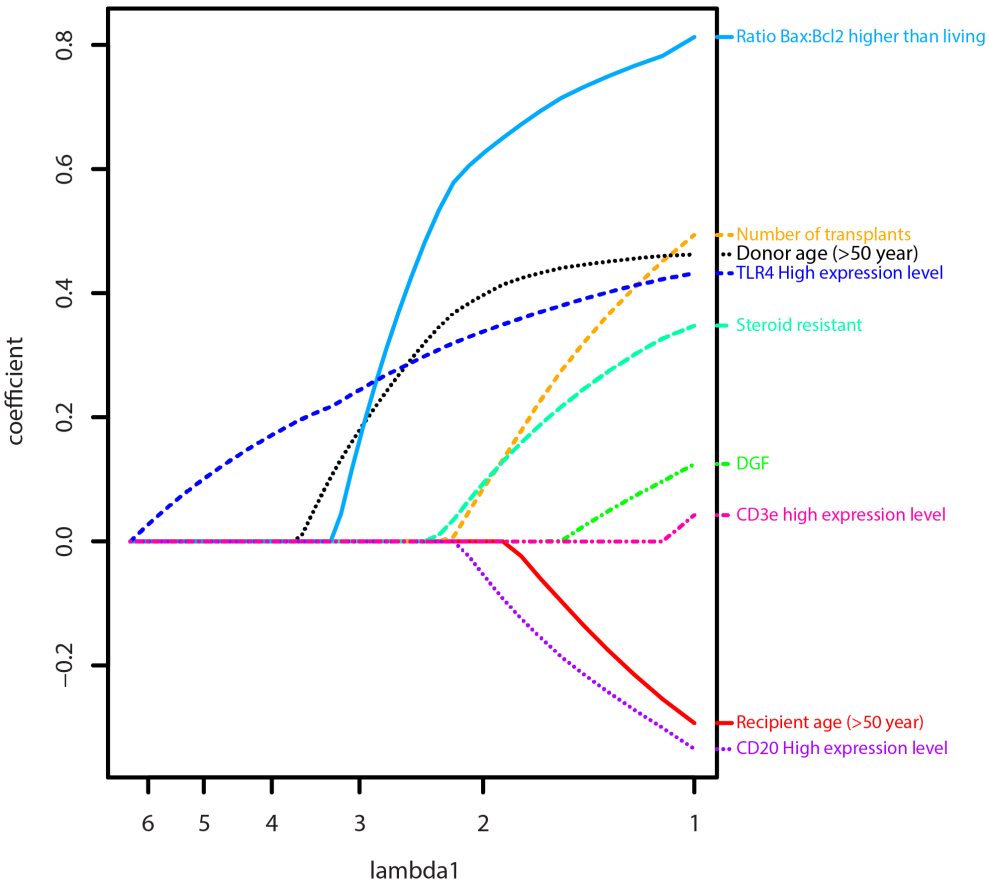


Figure S1. Penalized survival analysis of clinical risk factors. The plot shows the effect of lambda on the fitted regression coefficients. High TLR4 expression, donor age >50, and high Bax:Bcl2 ratio were the top three parameters in the penalized lasso model.

Table S1. Primer sequences, amplification efficiency and functional description

Genes	Forward primer (5'-3')	Reverse primer (5'-3')	Product size (bp)	Efficiency (%)	Description
TLR1	acaccaagttgtcagcgatg	gggctaattttggatgggca	135	94.5	Detects triacylated lipopeptides
TLR2	gtgataggtgtgaggcaggt	gtggccgccttgattcatag	136	100.8	Detects HSP70, HMGB1, HA
TLR3	gaacctccagcacaatgagc	tgacaagccattatgagacaga	169	99.6	Detects mRNA
TLR4	acctccccttctcaaccaag	ggctctgatatgccccatct	150	106.9	Detects HMGB1, heparan sulfate, S100A9, and LPS
TLR5	tgaagtctttaaacaaccaggga	tccaaacacaggaccggc	142	108.1	Detects flagellin
TLR6	ctctccactctgctttccca	ctccgaggactggtgcatat	109	107.1	Forms heterodimer with TLR2
TLR7	tgctctcttcaaccagacct	cacgatcacatggttctttgga	196	103.5	Detects RNA, ssRNA
TLR8	taccaccttgaagagagccg	tgctttggttgatgctctgc	112	99.5	Detects ssRNA
TLR9	agatggaggggagaaggtct	caaggtgaagttgagggtgc	112	98.6	Detects DNA
TLR10	tccagagctgccagaagaaa	aaatccagtgtcgttgtggc	102	97.5	Forms heterodimer with TLR2
CD46	attcagtgtggagtcgtgct	aattgtgtcgctgccatcg	167	95.8	Cofactor that inactivates C3 convertase
CD55	tcctggcgagaaggactcagtga	agccttgttggcacctcgca	96	98.0	Decay-accelerating activity C3 convertase
CD59	gcgtgtctcattaccaaagct	ccttcttgcagcagtagtacg	127	95.9	Prevents the formation of MAC
C2	tctgaacctctacctgctcc	acatgaggactttgggctct	164	92.8	Component in the classical and lectin pathway
C3	agcagtcaaggtctacgcc	ttgtccagccgttcttcca	180	95.3	Central role in complement activation
C4	ctgaacaaccgccagattcg	tggcaggtcgtgttcttcat	149	98.9	Component in the classical and lectin pathway
CR1	tgtttgcgatgaagggttcc	gagttcctgtgtgtctccca	155	101.4	Decay-accelerating activity and cofactor activity
Bcl2	gccctgtggatgactgagta	cttcagagacagccaggaga	135	100.4	Anti-apoptotic protein
BAX	cgccctttctactttgcca	ccaatgtccagcccatgatg	92	102.1	Pro-apoptotic protein
B-actin	accacaccttctacaatgag	tagcacagcctggatagc	161	94.0	Reference gene
GAPDH	acccactcctccacctttgac	tccaccaccctgttgctgtag	110	98.0	Reference gene
CD3e	ccgccatcttagtaaagtaacag	aataccacccatttcttcattacc	131	90	T cell marker
CD163	tggagtgacctgctcagatg	caccgtccttggaatttgat	99	103.8	M2 macrophage maker
CD68	ttcccctatggacacctcag	ttgtactccaccgccatgta	86	103.9	Pan macrophage maker
CD20	ggggctgtccagattatgaa	ccaggagtgatccggaaata	148	97.2	B cell marker

Table S2. Correlations of innate immunity markers with inflammatory markers and Banff lesions

		CD163	*CD68*	*CD20*	*CD3e*	g	i	t	v	ci	ct
TLR1	R	0.46	0.44	0.35	0.35	0.01	0.30	0.25	-0.02	0.13	0.16
	P	7.16E-8*	2.93E-7*	7.20E-5*	7.57E-5*	0.92	1.71E-03	0.01	0.86	0.18	0.09
TLR2	R	0.02	0.09	0.16	0.06	0.04	0.08	0.01	0.01	0.21	0.19
	P	0.84	0.34	0.07	0.53	0.67	0.40	0.91	0.90	0.03	0.05
TLR3	R	-0.19	-0.22	-0.11	0.05	-0.13	-0.21	-0.02	-0.11	-0.08	-0.04
	P	0.04	0.02	0.24	0.61	0.16	0.03	0.87	0.28	0.43	0.65
TLR4	R	0.48	0.52	0.16	0.23	0.10	0.11	0.19	0.12	0.13	0.20
	P	1.73E-8*	8.83E-10*	0.08	0.01	0.29	0.25	0.04	0.22	0.17	0.04
TLR5	R	0.21	0.09	0.24	0.15	-0.08	0.11	0.08	0.02	0.16	0.19
	P	0.02	0.35	0.01	0.1	0.43	0.23	0.39	0.83	0.10	0.05
TLR6	R	0.08	0.1	0.32	0.11	-0.02	0.13	0.09	-0.05	0.24	0.20
	P	0.38	0.25	3.97E-4*	0.22	0.88	0.16	0.38	0.65	0.01	0.04
TLR7	R	0.44	0.52	0.45	0.44	0.03	0.23	0.33	-0.18	0.12	0.07
	P	2.62E-7*	1.12E-9*	1.80E-7*	2.77E-7*	0.77	0.02	4.66E-4	0.08	0.22	0.45
TLR8	R	0.42	0.51	0.17	0.18	0.22	0.27	0.24	0.05	0.17	0.13
	P	1.01E-6*	1.33E-9*	0.06	0.05	0.02	0.00	0.01	0.59	0.08	0.17
TLR9	R	-0.1	-0.05	0.37	0.1	-0.12	0.11	0.10	-0.18	0.25	0.13
	P	0.25	0.58	2.70E-5*	0.26	0.20	0.24	0.31	0.08	0.01	0.18
TLR10	R	-0.1	-0.05	0.5	0.17	-0.11	0.18	0.14	-0.13	0.20	0.09
	P	0.27	0.56	6.87E-9*	0.05	0.27	0.07	0.16	0.21	0.04	0.37
CD46	R	-0.48	-0.47	-0.11	-0.07	-0.11	-0.19	-0.11	-0.23	-0.19	-0.04
	P	1.92E-8*	3.61E-8*	0.25	0.46	0.26	0.04	0.27	0.02	0.05	0.69
CD55	R	0.2	0.1	0.11	0.03	0.03	0.03	1.85E-4	0.07	0.04	0.28
	P	0.03	0.26	0.23	0.73	0.74	0.72	1.00	0.50	0.65	3.07E-3

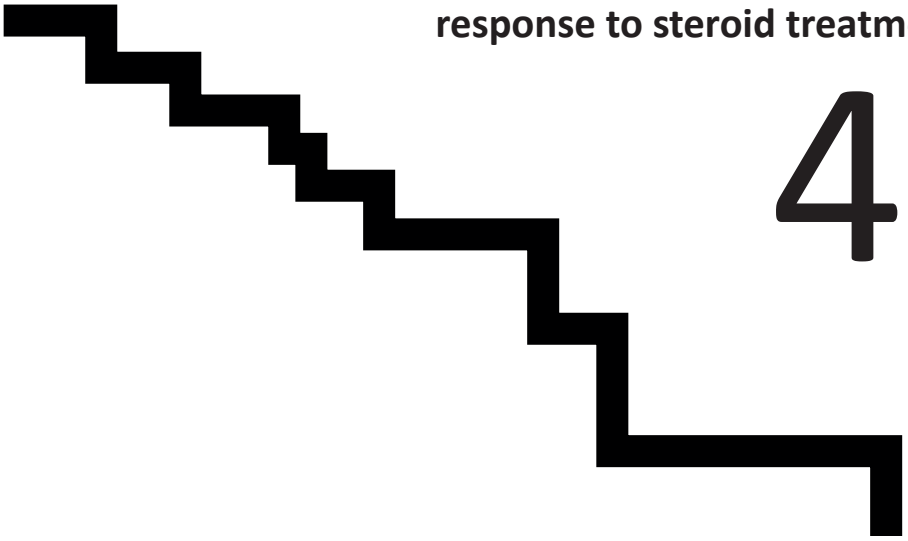
Table S2: Continued

CD59	R	-0.37	-0.36	-0.3	-0.18	0.15	-0.17	-0.15	0.02	-0.14	-4.32E-3
	P	3.03E-5*	4.66E-5*	7.912E-04	0.05	0.11	0.07	0.13	0.84	0.15	0.96
C2	R	0.64	0.68	0.21	0.42	0.07	0.32	0.32	0.13	0.29	0.20
	P	3.12E-15*	9.04E-18*	0.02	1.74E-6*	0.50	7.82E-4	7.76E-4	0.19	2.79E-3	0.04
C3	R	0.4	0.4	0.26	0.22	0.09	0.33	0.10	0.08	0.31	0.17
	P	5.41E-6*	6.71E-6*	0.01	0.02	0.34	5.85E-4	0.30	0.45	1.08E-3	0.09
C4	R	-0.23	-0.18	0.05	0.08	-0.14	0.06	0.09	-0.15	-0.07	0.04
	P	0.01	0.05	0.59	0.38	0.14	0.51	0.36	0.14	0.45	0.70
CR1	R	0.7	0.52	0.43	0.33	0.00	0.44	0.27	0.12	0.25	0.26
	P	6.53E-19*	1.16E-9*	8.98E-7*	1.96E-4*	0.96	1.59E-6*	4.18E-3	0.22	0.01	0.01
BCL2	R	-0.28	-0.27	0.22	0.15	-0.26	-0.08	0.06	-0.05	-0.07	-0.08
	P	2.20E-03	2.30E-03	0.02	0.09	0.01	0.42	0.53	0.66	0.47	0.40
BAX	R	0.16	0.04	0.11	0.1	-0.05	0.03	0.03	0.12	0.16	0.13
	P	0.07	0.7	0.24	0.29	0.59	0.79	0.74	0.24	0.10	0.19
BAX:BCL2	R	0.45	0.33	-0.18	-0.18	0.24	0.07	-0.05	0.15	0.24	0.17
	P	2.13E-7*	2.42E-4*	0.06	0.05	0.01	0.47	0.64	0.14	0.01	0.09

g, glomerulitis; i, interstitial inflammation; t, tubulitis; v, intimal arteritis; ci, interstitial fibrosis; ct; tubular atrophy; R, Spearman Correlation Coefficient; P, P value.

* Statistically significant p-values after Bonferroni correction (P<0.00025).

Endothelial-epithelial-related transcriptional levels in acute rejection biopsies of kidney transplant recipients are predictive for a worse response to steroid treatment



4

Jianxin Yang, Marijke Spruyt-Gerritse, Jacqueline Anholts,
Frans H.J. Claas, Michael Eikmans*

Department of Immunohematology and Blood Transfusion,
Leiden University Medical Center, Leiden, the Netherlands.

Abstract

Background: Molecular assessment of transplant biopsies may help in prognostic assessment. We investigated whether transcriptional alterations in acute rejection biopsies provide information on outcome.

Method: We studied a cohort of patients transplanted between 1995 and 2005. Eighty-five biopsies taken due to clinical acute rejection (median 14 days [9.5-35.0] post-transplant) were analyzed for 23 T-cell mediated rejection (TCMR)-related transcripts (including CD28, lag-3, CD8a, granulysin, ICOS, BTLA) and 13 endothelium-epithelium-related transcripts (including PECAM1, ICAM2, von Willebrand factor, E-selectin, CD34, caveolin 1) using the Fluidigm high throughput RT-PCR system.

Results: TCMR transcripts as well as the endothelium-epithelium-related transcripts clustered together in principal component analysis. Therefore, for each patient a composite score, resulting in a T-score and E-score, respectively, was calculated. Five control transplant biopsies, containing no morphologic abnormalities, showed a profile of high E-score and low T-score.. In the low E-score group with rejection (n=46), 52.2% of the patients showed resistance to steroid treatment, whereas in the high E-score group (n=39) this was 28.2% ($P<0.05$). Both the T-score and rejection severity according to Banff criteria were not significantly associated with response to steroid treatment.

Conclusion: A molecular signature of relatively low transcripts levels of endothelium-epithelium-related genes may reflect injury of the microvasculature in the allografts, posing a risk factor for decreased therapy response. The results suggest that molecular assessment of the graft tissue has an added value to histomorphologic evaluation.

Introduction

Acute allograft rejection remains a risk factor for adverse transplant outcome (1). Rejection is associated with infiltration of host inflammatory cells and allograft injury. Histologic assessment according to the Banff classification is used to determine the type of rejection, but may be of limited prognostic value.

Gene expression assessment in the biopsy tissue may represent an objective means of analysis and a complementary tool to conventional diagnostic measurements. Numerous studies have described molecular markers in blood, urine, and graft tissue, which are associated with acute rejection (2-7). Prediction of the therapeutic response to steroid treatment remains difficult using only clinical and histomorphologic parameters. Sarwal and colleagues reported that patients with steroid resistant rejection display elevated expression of T cell, natural killer cell, and B cells (3). Subsequent studies did not show an association of B cell infiltrates with steroid resistant rejection and graft function (8-10). The extent of staining of C4d, CD68, HLA-DR, and granulysin, and an elevated expression of Fas ligand in the graft tissue were associated with steroid resistance rejection (2). Rekers and colleagues showed increased expression of metallothioneins and LAG-3 and CD25:CD3e ratio in biopsy samples associated with steroid resistant rejection (11, 12).

Microvascular injury is associated with rejection: preservation of microvascular integrity and absence of inflammation are important for maintaining long term graft function (13, 14). Nickeleit et al reported that endarteritis, defined as the presence of inflammatory cell in the sub-endothelial space and adherence of mononuclear cell to endothelial cells, is associated with steroid resistant rejection (15). Haas et al confirmed that especially type 2B rejection (severe intimal arteritis comprising >25% of the luminal area) leads to a worse response to steroid therapy (16). Ozdemir et al showed that destruction of the microvasculature, as reflected by loss of endothelial markers, is associated with steroid resistant rejection (17).

In the current study, we examined 23 TCMR and 13 endothelium-epithelium related transcripts using the Fluidigm high throughput RT-PCR system in acute rejection renal biopsies, and investigated their relation to clinical outcome.

Materials and methods

Patient characteristics

Patients who underwent kidney transplantation at the Leiden University Medical Center (LUMC) between 1995 and 2005 were investigated. Biopsy samples were taken within 6 months after transplantation from 85 patients with clinical suspicion of acute rejection and 5 patients without histological rejection. Immunofluorescent C4d staining on 80

biopsy samples were performed as described previously (8). Donor specific antibody (DSA) information was not routinely available in this study. Biopsy samples were assessed blindly by two pathologists according to Banff 2011 criteria (11, 18). Informed consent was obtained from all individuals. Patient characteristics are shown in Table 1.

Selection of genes

Genes included for expression profiling were selected from previous studies. The endothelium-epithelium-related transcripts were selected based on probes that were differentially expressed between endothelial cells and non-endothelial cells (19), and between biopsies with ABMR and other biopsies (20, 21): CDH13, PLA1A, ROBO4, TM4SF18, GNG11, PGM5, KLF4, CAV1, CDH5, vWF, CD34, PECAM1, MCAM. The set of TCMR related molecules contained transcripts that were increased highest in expression in T cell mediated rejection biopsies compared to other transplant biopsies (22, 23): SLAMF8, TNFSF8, CD96, SIRPG, BTLA, SLA, ANKRD22, CD28, CD274, SP140, SH2D1A, ADAMDEC1, IL12RB1, LAG3, PTPN7, CD72, CD8a, CXCL13, CXCL10, GNLY, ICOS, RARRES3, TOX2. Majority of the primer sets were designed to target separate exons, spanning at least one intron (> 800 bp) to prevent amplification of genomic DNA.

RNA extraction and cDNA synthesis

RNA isolation and quality check were performed as described previously (12). Total RNA (50-200 ng) was used for cDNA synthesis, following the manufacturer's manuals.

High throughput qPCR analysis using Fluidigm 96.96 dynamic array

Ten times diluted cDNA (1.25 uL) was pre-amplified containing 2.5 uL of Taqman Preamp master mix (Applied Biosystems, Texas, USA) and 1.25 uL of pooled primer mix for 14 cycles. The qPCR reactions were performed using Eva-green dye following the Fluidigm protocol, and results were collected on the BioMark HD system. Absolute Cq values from duplicate measurements of each transcript were averaged, and relative gene expression levels were normalized to the geometric mean of the reference genes glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-actin.

Statistical analyses

Relative expression level were log2 transformed and normalized using the z-score for each transcript. The normalized z-score were averaged for each distinct set of transcripts and for each patient. Frequency of patients with steroid resistant rejection in high and low score groups were analyzed by chi-square test. Difference in score of individual endothelial related transcript between steroid responsive and resistant rejection were analyzed using T-test. Death-censored graft survival curves were created using the Kaplan-Meier method, and differences between curves were calculated using log rank tests.

Results

Demographics and clinical data

We studies 85 biopsy samples from patients with a clinical indication of acute rejection and 5 protocol biopsies without any indication of rejection. Acute rejection were treated by intravenous methylprednisolone. Thirty five patients had a steroid-resistant rejection, and fifty had a steroid responsive rejection. Steroid resistance was defined as no response to steroid treatment and requirement for antithymocyte globulin therapy within 14 days after the start of the steroid treatment, as described previously (11, 12). Clinical parameters and histologic lesions were not associated with steroid response treatment (Table S1).

Table 1. Demographics of patient cohort.

Variable	Number (%)
Recipient age (>= 50 year)	37 (43.5%)
Recipient gender (Female)	27 (31.8%)
Donor age (>= 50 year)	34 (40%)
Donor gender (Female)	55 (64.7%)
Donor type (Living)	20 (23.5%)
Time from transplant to rejection (days, IQR)	14 (9.5-35)
First transplantation (Yes)	71 (83.5%)
HLA AB-matching (Yes)	12 (14.1%)
HLA DR-matching (Yes)	25 (29.4%)
DGF in deceased donor (Yes)	23 (35.4%)
Steroid responsiveness	50 (58.8%)
Cold ischemia time (<= 18 h)	19 (22.4%)
Induction therapy (IL-2R blocker)	26 (30.6%)
Maintenance therapy	
Corticosteroid, CNI	34 (40%)
Corticosteroid, MMF	4 (4.7%)
Corticosteroid, CNI, MMF	34 (40%)
Banff score	
Glomerulitis (g=0/1/2/3)	56/20/3/2
Interstitial inflammation (i=1/2/3)	36/27/18
Tubulitis (t=0/1/2/3)	3/29/32/17
Intimal arteritis (v=0/1/2/3)	44/20/4/6
Interstitial fibrosis (ci=0/1/2)	51/24/6
Tubular atrophy (ct=0/1/2)	47/32/2
C4d staining (diffuse positive)	11(13.8%)
Graft survival (Death censored)	
>1 year	81 (95.3%)
>6 year	79 (92.9%)

Eleven (13.8%) biopsies showed diffuse C4d positive staining. The median (IQR) time of biopsy was 14 (9.5-35) days post-transplant. Twenty-six (30.6%) patients received IL-2 receptor blocker monoclonal antibody as induction therapy. Thirty-eight patients (44.7%) received a double drug regime (corticosteroid, calcineurin inhibitor or MMF) and thirty-four patients (40%) received a triple drug regime (corticosteroid, calcineurin inhibitor, and mycophenolate mofetil) as maintenance immunosuppressive therapy (Table 1).

HLA, human leukocyte antigen; DGF, delayed graft function; IQR, Interquartile ranges; CNI, calcineurin inhibitor; MMF, mycophenolate mofetil.

Steroid resistant rejection predicts inferior long term graft outcome

The effect of steroid resistant rejection on long term graft survival was assessed. Patients showing a poor response to steroid treatment had inferior long term graft survival (66.4%) compared to patients with a steroid-responsive rejection (95.4%, P=0.022, Figure 1).

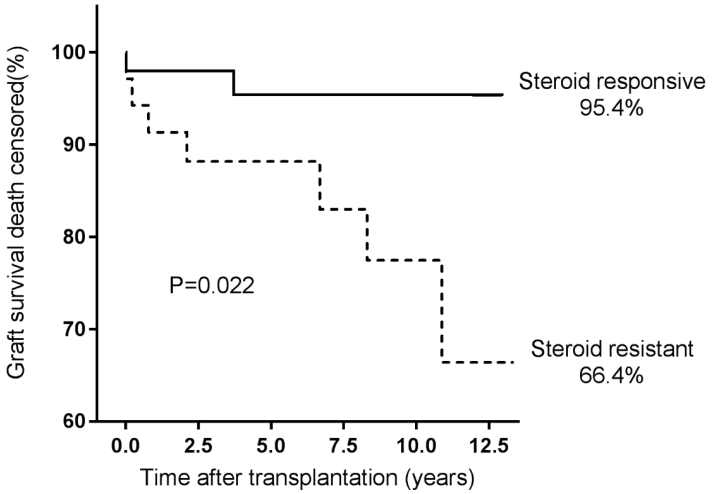


Figure 1. Association of steroid resistant with long term graft survival. Steroid resistant rejection were significantly related with inferior graft survival after kidney transplant.

Fluidigm dynamic array produce consistent result with conventional qPCR.

RNA from the transplant biopsies with acute rejection was subjected to gene expression assessment by the Fluidigm dynamic array system. To validate this system, two transcripts were additionally tested using conventional qPCR. The absolute Cq values highly correlated between Fluidigm dynamic array and conventional qPCR (R^2>0.92), indicating that the Fluidigm dynamic array generated comparable results with conventional real time PCR (Figure S1).

Transcripts were clustered into two groups

The normalized gene expression data were analyzed based on principal component analysis (PCA). Both the endothelial-epithelial related transcripts (E-group) and TCMR related transcripts (T-group) clustered together by the first component of PCA (Figure 2). Based on this observation, we decided to calculate for each patient the composite score of the endothelial-epithelial related transcripts (termed as E-score) and of the TCMR related group transcripts (termed as T-score).

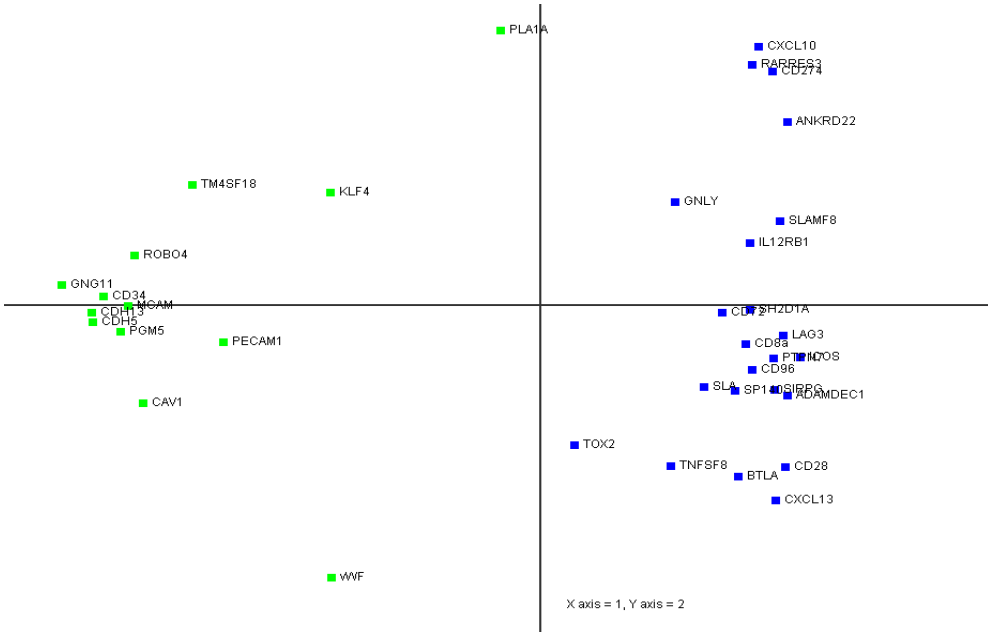


Figure 2. Principal component analysis for clustering the transcripts. The first component could divide the transcripts into two groups except the E-selectin: Endothelial-epithelial related group (green dots) and T cell mediated rejection related group (blue dots).

E-score associated with steroid resistant rejection

The patient group with acute rejection was divided into two groups based on the E-score: E-high (E-score>0, n=39) and E-low (E-score<0, n=46). In the E-low group 52.2% of the patients showed resistance to steroid treatment, which was significantly higher than the 28.2% of patients in the E-high group (P=0.025, Figure 3 and Table 2). The E-score was not associated with any of the Banff lesions (Table S2).

Table 2. Relationship between molecular scores and steroid response treatment.

	Steroid responsive (N=50)	Steroid resistant (N=35)	P
E-score			0.025*
E-score<0	22 (47.2%)	24 (52.2%)	
E-score>0	28 (71.8%)	11 (28.2%)	
T-score			
T-score<0	18 (62.1%)	11 (37.9%)	0.662
T-score>0	32 (57.1%)	24 (42.9%)	

*P values were calculated using Chi-Square test.

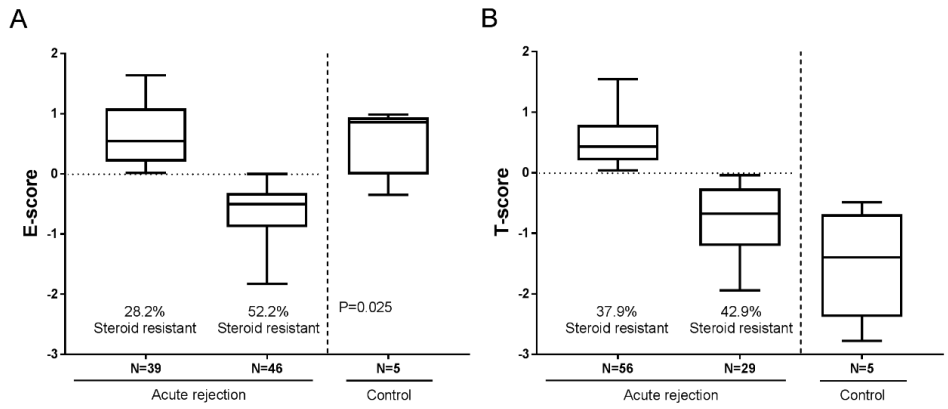


Figure 3. Molecular score associated with steroid resistant rejection. In the E-low group 52.2% of the patients showed resistance to steroid treatment, which was significantly higher than the 28.2% of patients in the E-high group. The T-high and T-low group were not significantly different in the percentage of patients having steroid resistant rejection

The association of individual endothelial related transcript with steroid resistant rejection was analyzed. Patients with steroid resistant rejection showed significantly lower expression of TM4SF18, PGM5, and CD34 compared to patients with steroid response treatment after multiple correction (Table 3).

The patients without rejection showed relatively high E-score and low T-score (Figure 3).

T-score not associated with steroid resistant rejection but was related to Banff inflammation score

The patient group with acute rejection was divided into two groups based on the T-score: T-high (T-score>0, n=56) and T-low (T-score<0, n=29). The T-high and T-low group were not significantly different in the percentage of patients having steroid resistant rejection (37.9% versus 42.9%, Figure 3 and Table 2).

The T-score was significantly correlated with interstitial inflammation and tubulitis (Table S2). The frequency of patients with i>1 was 71.2% in the T-high group, and 27.6% in the T-low group (P<0.005). Similarly, 82.7% of patients in the T-high group had moderate or severe tubulitis (t>1) in contrast to 20.7% in the T-low group (P<0.0001). The T-high group also had significantly higher incidence of interstitial fibrosis (46.1%) than the T-low group (26.7%, P=0.048). The T-score was not associated with intimal arteritis.

Table 3. Association between individual endothelial-epithelial related transcripts and steroid response treatment.

	Steroid responsive (N=50)	Steroid resistant (N=35)	P
CAV1	0.231 ± 0.796	-0.345 ± 1.204	0.017
CD34	0.204 ± 0.856	-0.500 ± 0.972	0.001*
CDH13	0.157 ± 0.893	-0.344 ± 1.093	0.023
CDH5	0.146 ± 0.938	-0.340 ± 1.018	0.026
GNG11	0.189 ± 0.956	-0.417 ± 0.900	0.004
KLF4	0.233 ± 1.119	-0.289 ± 0.746	0.018
MCAM	0.137 ± 0.909	-0.282 ± 1.098	0.058
PECAM1	0.234 ± 0.911	-0.341 ± 1.046	0.008
PGM5	0.193 ± 0.923	-0.447 ± 0.925	0.002*
PLA1A	0.131 ± 0.925	-0.213 ± 1.105	0.122
ROBO4	0.171 ± 0.933	-0.404 ± 0.969	0.007
TM4SF18	0.236 ± 0.897	-0.473 ± 0.968	0.001*
vWF	0.173 ± 0.809	-0.100 ± 1.040	0.178

[a] Gene expression data shown as mean ± SD.

*P values were calculated by T-test and adjusted by Bonferroni method (P<0.00385).

Discussion

In the present study mRNA expression levels of endothelium-epithelium related transcripts (E-score) and T-cell mediated rejection related transcripts (T-score) were investigated in transplant biopsies at time of acute rejection (AR). We found that a relatively low E-score is associated with resistance to steroid treatment, whereas the T-score and Banff score were not related to outcome of the rejection. The T-score is significantly associated with interstitial inflammation and tubulitis. Results from this study suggest that molecular assessment offer an added value to histologic diagnosis with respect to predicting of steroid therapy.

Evaluation of multiple markers that belong to a similar pathophysiologic pathway is superior to tests of single markers, as it may decrease the variation introduced by aberrant expression and by inter-laboratory differences. The Fluidigm dynamic arrays system provides a high throughput gene expression platform on the basis of real time quantitative PCR, which requires very low input amounts of nucleic acid. Our data, consistent with other studies, show that the microfluidic technology has high concordance with conventional qPCR (24, 25). The automated microfluidic chip system enables faster analysis, and it significantly reduces the reagent and sample consumption (26).

T-group transcripts, previously described to be elevated in TCMR biopsy samples compared to all other conditions (23), mainly reflect T cell co-stimulation, activation and signalling, and cytotoxic T cell- and INF-γ-related effects. Majority of the biopsies obtained in the previous study were taken more than one year after transplantation (6). We interrogated these transcripts in a cohort of biopsies, containing acute rejection, most of which had been taken within 3 months after transplantation). Consistent with a previous study (22), the relative high T-score was significantly associated with the extent of interstitial inflammation and tubulitis, but not with intimal arteritis. Thus, assessment of T-score in biopsy samples provides additional value on diagnosis of TCMR.

Here we found that decreased expression of endothelium and epithelium related transcripts is associated with resistance to steroid pulse treatment in kidney transplantation. In contrast, Sis et al reported that endothelial associated transcriptional levels were elevated in late biopsies diagnosed with ABMR (21). Sellares et al subsequently demonstrated that molecular transcripts that typified biopsies with ABMR were mainly expressed in endothelial, epithelial cells, and NK cells, which was confirmed by another prospective study (20, 27). The seemingly contradicting clinical effect of dysregulated expression of endothelial cell transcripts between previous studies and ours may be explained by a difference in the time period of the biopsies after transplantation (early versus late), and the type of the rejections studied. Our cohort mostly contained TCMRs on the basis of histomorphology. Unfortunately, we cannot completely rule out a humoral component for the minority of cases that showed C4d positivity, since serum for donor specific antibody screening at time of rejection was not always available.

Endothelial cells in glomeruli and peritubular capillaries mediate critical processes such as inflammation and coagulation. Decreased expression of endothelium and epithelium related transcripts, which are involved in blood vessel development and biological adhesion, may reflect low nephron integrity and reduced nephron repair after injury. The analysis of individual transcripts showed that TM4SF18, PGM5, and CD34 remain significantly lower in patients with steroid resistant rejection after Bonferroni correction. TM4SF18 is a member of transmembrane 4 large six family, characterized by four conserved transmembrane domains (28). The tetraspanins can form a large protein complex with integrin and growth receptors, which may mediate cell adhesion, proliferation, and migration (29, 30). PGM5 is involved in cell-to-cell adherens junctions of endothelial and epithelial cells (31-33). CD34 is mainly expressed on endothelium, epithelium, and human hematopoietic stem cells, and it is a potential marker of endothelial progenitor cells (34). In combination with MCAM, PECAM1, CAV1, ROBO4, PGM5, and cadherin molecules, these molecules are involved in angiogenesis during wound healing and in cell adhesion, all in the context of enhanced repair capacity and kidney integrity. Clinically this may mean that the renal function is more likely to recover after high-dose steroid therapy when the kidney has a higher repair capacity and nephron integrity, as reflected by the relatively high expression of the endothelial transcripts

studied. This notion is supported by the observation that severe vascular destruction is related to worse response to steroid treatment, compared to mild vascular destruction (17). Furthermore, severe intimal arteritis and adherence of mononuclear cells to endothelial cells were associated with steroid resistant rejection (15, 16).

In conclusion, we found that decreased expression of endothelial-epithelial transcripts in the biopsy during acute rejection, as reflected in a low E-score, is significantly associated with a poor response to steroid treatment. The expression of TCMR-related transcripts, as reflected in the T-score, and Banff lesions in the biopsy were not associated with steroid response. Molecular assessment of biopsies at moment of rejection may provide additional support for clinical diagnosis. The prognostic value of the expression profiles studied for predicting steroid resistant rejection would need to further tested in a prospective study.

Acknowledgments

J. Yang was awarded financial support from the China Scholarship Council (201306170038).

Reference

1. Pallardo Mateu LM, Sancho Calabuig A, Capdevila Plaza L, Franco Esteve A. Acute rejection and late renal transplant failure: risk factors and prognosis. Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association. 2004;19 Suppl 3(suppl 3):iii38-42.
2. Eikmans M, Roelen DL, Claas FH. Molecular monitoring for rejection and graft outcome in kidney transplantation. Expert opinion on medical diagnostics. 2008;2(12):1365-79.
3. Sarwal M, Chua MS, Kambham N, Hsieh SC, Satterwhite T, Masek M, et al. Molecular heterogeneity in acute renal allograft rejection identified by DNA microarray profiling. The New England journal of medicine. 2003;349(2):125-38.
4. Li B, Hartono C, Ding R, Sharma VK, Ramaswamy R, Qian B, et al. Noninvasive diagnosis of renal-allograft rejection by measurement of messenger RNA for perforin and granzyme B in urine. New England Journal of Medicine. 2001;344(13):947-54.
5. Roedder S, Sigdel T, Salomonis N, Hsieh S, Dai H, Bestard O, et al. The kSORT assay to detect renal transplant patients at high risk for acute rejection: results of the multicenter AART study. PLoS medicine. 2014;11(11):e1001759.
6. Halloran PF, Reeve J, Akalin E, Aubert O, Bohmig GA, Brennan D, et al. Real time central assessment of kidney transplant indication biopsies by microarrays: the INTERCOMEX study. American Journal of Transplantation. 2017.
7. Suthanthiran M, Schwartz JE, Ding R, Abecassis M, Dadhania D, Samstein B, et al. Urinary-cell mRNA profile and acute cellular rejection in kidney allografts. New England Journal of Medicine. 2013;369(1):20-31.
8. Eikmans M, Roos-van Groningen MC, Sijpkens YW, Ehrchen J, Roth J, Baelde HJ, et al. Expression of surfactant protein-C, S100A8, S100A9, and B cell markers in renal allografts: investigation of the prognostic value. Journal of the American Society of Nephrology. 2005;16(12):3771-86.
9. Scheepstra C, Bemelman FJ, van der Loos C, Rowshani AT, Idu MM, ten Berge IJ, et al. B cells in cluster or in a scattered pattern do not correlate with clinical outcome of renal allograft rejection. Transplantation. 2008;86(6):772-8.
10. Kayler LK, Lakkis FG, Morgan C, Basu A, Blisard D, Tan HP, et al. Acute cellular rejection with CD20-positive lymphoid clusters in kidney transplant patients following lymphocyte depletion. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons. 2007;7(4):949-54.
11. Rekers NV, Bajema IM, Mallat MJ, Anholts JD, de Vaal YJ, Zandbergen M, et al. Increased metallothionein expression reflects steroid resistance in renal allograft recipients. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons. 2013;13(8):2106-18.
12. Rekers NV, Bajema IM, Mallat MJ, Zuidwijk K, Anholts JD, Goemaere N, et al. Quantitative polymerase chain reaction profiling of immunomarkers in rejecting kidney allografts for predicting response to steroid treatment. Transplantation. 2012;94(6):596-602.
13. Bishop GA, Waugh JA, Landers DV, Krensky AM, Hall BM. Microvascular destruction in renal transplant rejection. Transplantation. 1989;48(3):408-14.
14. Contreras AG, Briscoe DM. Every allograft needs a silver lining. The Journal of clinical investigation. 2007;117(12):3645-8.
15. Nickeleit V, Vamvakas EC, Pascual M, Poletti BJ, Colvin RB. The prognostic significance of specific arterial lesions in acute renal allograft rejection. Journal of the American Society of Nephrology : JASN. 1998;9(7):1301-8.
16. Haas M, Kraus ES, Samaniego-Picota M, Racusen LC, Ni W, Eustace JA. Acute renal allograft rejection with intimal arteritis: histologic predictors of response to therapy and graft survival. Kidney Int. 2002;61(4):1516-26.
17. Özdemir BH, Demirhan B, Özdemir FN, Dalgiç A, Haberal M. The role of microvascular injury on steroid and OKT3 response in renal allograft rejection. Transplantation. 2004;78(5):734-40.
18. Mengel M, Sis B, Haas M, Colvin RB, Halloran PF, Racusen LC, et al. Banff 2011 Meeting report: new concepts in antibody-mediated rejection. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons. 2012;12(3):563-70.

19. Ho M, Yang E, Matcuk G, Deng D, Sampas N, Tsalenko A, et al. Identification of endothelial cell genes by combined database mining and microarray analysis. Physiol Genomics. 2003;13(3):249-62.
20. Sellares J, Reeve J, Loupy A, Mengel M, Sis B, Skene A, et al. Molecular diagnosis of antibody-mediated rejection in human kidney transplants. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons. 2013;13(4):971-83.
21. Sis B, Jhangri GS, Bunnag S, Allanach K, Kaplan B, Halloran PF. Endothelial gene expression in kidney transplants with alloantibody indicates antibody-mediated damage despite lack of C4d staining. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons. 2009;9(10):2312-23.
22. Reeve J, Sellares J, Mengel M, Sis B, Skene A, Hidalgo L, et al. Molecular diagnosis of T cell-mediated rejection in human kidney transplant biopsies. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons. 2013;13(3):645-55.
23. Venner J, Famulski K, Badr D, Hidalgo L, Chang J, Halloran P. Molecular Landscape of T Cell–Mediated Rejection in Human Kidney Transplants: Prominence of CTLA4 and PD Ligands. American Journal of Transplantation. 2014;14(11):2565-76.
24. Spurgeon SL, Jones RC, Ramakrishnan R. High throughput gene expression measurement with real time PCR in a microfluidic dynamic array. PloS one. 2008;3(2):e1662.
25. Jang JS, Simon VA, Feddersen RM, Rakhshan F, Schultz DA, Zschunke MA, et al. Quantitative miRNA expression analysis using fluidigm microfluidics dynamic arrays. BMC genomics. 2011;12(1):144.
26. Liu J, Hansen C, Quake SR. Solving the "world-to-chip" interface problem with a microfluidic matrix. Analytical chemistry. 2003;75(18):4718-23.
27. Halloran PF, Pereira AB, Chang J, Matas A, Picton M, De Freitas D, et al. Microarray diagnosis of antibody-mediated rejection in kidney transplant biopsies: an international prospective study (INTERCOM). American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons. 2013;13(11):2865-74.
28. Wright MD, Ni J, Rudy GB. The L6 membrane proteins—a new four-transmembrane superfamily. Protein Science. 2000;9(8):1594-600.
29. Detchokul S, Williams ED, Parker MW, Frauman AG. Tetraspanins as regulators of the tumour microenvironment: implications for metastasis and therapeutic strategies. British Journal of Pharmacology. 2014;171(24):5462-90.
30. Lee JW. Transmembrane 4 L Six Family Member 5 (TM4SF5)-Mediated Epithelial–Mesenchymal Transition in Liver Diseases. International review of cell and molecular biology. 319: Elsevier; 2015. p. 141-63.
31. Edwards YH, Putt W, Fox M, Ives JH. A novel human phosphoglucomutase (PGM5) maps to the centromeric region of chromosome 9. Genomics. 1995;30(2):350-3.
32. Belkin AM, Klimanskaya IV, Lukashev ME, Lilley K, Critchley DR, Koteliansky VE. A Novel Phosphoglucomutase-Related Protein Is Concentrated in Adherens Junctions of Muscle and Nonmuscle Cells. Journal of Cell Science. 1994;107(1):159-73.
33. Belkin AM, Burridge K. Expression and localization of the phosphoglucomutase-related cytoskeletal protein, aciculin, in skeletal muscle. J Cell Sci. 1994;107 (Pt 7)(7):1993-2003.
34. Yoder MC. Human endothelial progenitor cells. Cold Spring Harb Perspect Med. 2012;2(7):a006692.

Supplementary Data

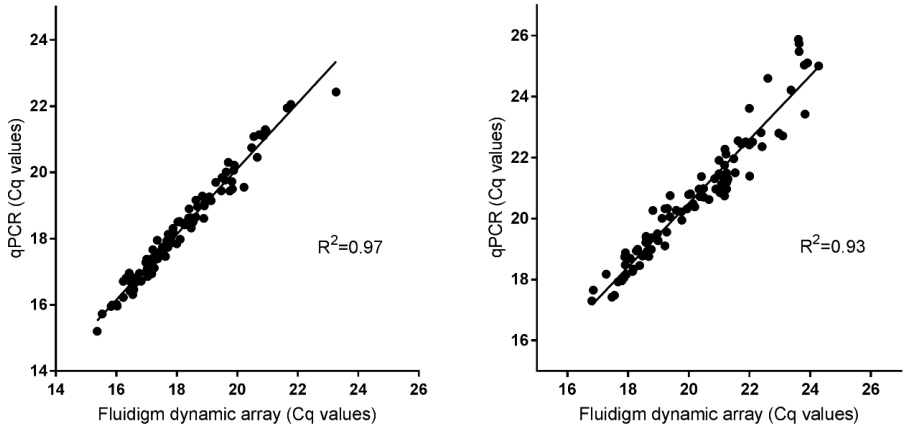


Figure S1. Absolute Cq values of Fluidigm dynamic array and conventional real time PCR. The highly correlated results suggested comparable results between Fluidigm technique and conventional qPCR .

Table S1. Clinical parameters and histologic lesions were not associated with steroid resistant rejection

	Steroid responsive (N=50)	Steroid resistant (N=35)	P
Recipient age (years, IQR)	48.5 (36.75-53)	47 (37-55)	0.96
Donor age (years, IQR)	47 (32.5-56.5)	46 (36-59)	0.58
Donor type			0.15
Living	9	11	
Cadaveric	41	24	
First transplantation [a]			0.72
Yes	42	29	
No	7	6	
HLA-ABDR matching [a]			1
Full Matching	5	4	
Mismatching	44	31	
DGF in deceased donor			0.42
Yes	16	7	
No	25	17	
Glomerulitis (g)[b]			0.37
0	35	21	
1	11	9	
2	2	1	
3	0	2	
Interstitial inflammation (i)[b]			0.12
1	20	16	
2	20	7	
3	8	10	
Tubulitis (t)[b]			0.39
0	2	1	
1	18	11	
2	21	11	
3	7	10	
Interstitial fibrosis (ci)[b]			0.52
0	30	21	
1	13	11	
2	5	1	
Tubular atrophy (ct)[b]			0.96
0	28	19	
1	19	13	
2	1	1	
Intimal arteritis (v)[c]			0.46
0	30	14	
1	10	10	
2	2	2	
3	3	3	

[a, b, c] data missing for one, four, eleven patient

*P values were calculated using Chi-Square test or Fisher's Exact Test.

Table S2. Relationship between group score and histologic lesions

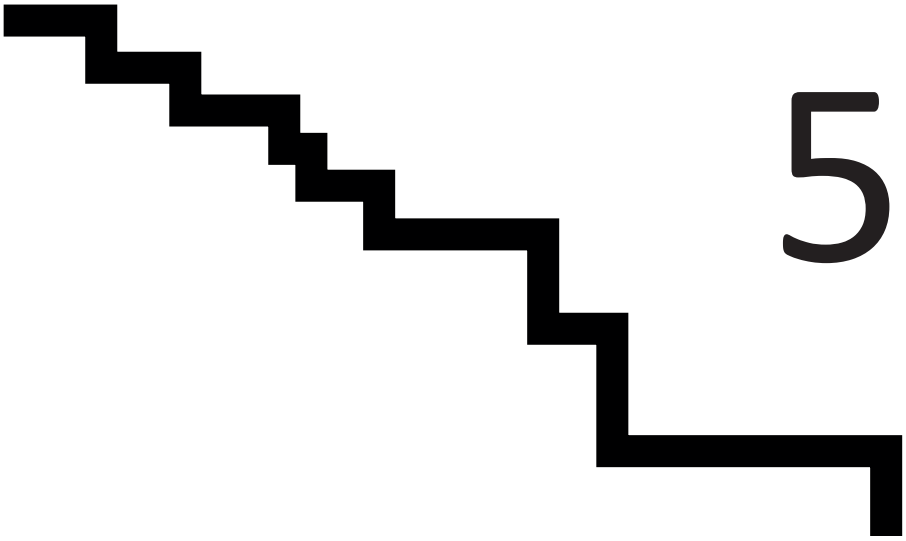
	E-score<0 (N=43)	E-score>0 (N=38)	P	T-score<0 (N=29)	T-score>0 (N=52)	P
Glomerulitis (g)			0.88			0.13
0	31	25		16	40	
1	10	10		11	9	
2	1	2		1	2	
3	1	1		1	1	
Interstitial inflammation (i)			0.60			5.84E-4*
1	17	19		21	15	
2	15	12		6	21	
3	11	7		2	16	
Tubulitis (t)			0.54			5.88E-8*
0	1	2		2	1	
1	13	16		21	8	
2	18	14		6	26	
3	11	6		0	17	
Interstitial fibrosis (ci)			0.43			0.048*
0	29	22		23	28	
1	10	14		4	20	
2	4	2		2	4	
Tubular atrophy (ct)			0.29			0.75
0	27	20		18	29	
1	16	16		10	22	
2	0	2		1	1	
Intimal arteritis (v) [a]			0.52			0.92
0	25	19		14	30	
1	9	11		7	13	
2	3	1		2	2	
3	2	4		2	4	

[a]data missing for seven patients

E-score, Endothelial-epithelial group score; T-score, TCMR related group score

P values were calculated using Chi-Square test or Fisher's Exact Test.

Genome-wide association study of acute rejection in kidney transplantation



5

Jianxin Yang[1], Geertje J. Dreyer[2], Jessica van Setten[3], Hans W. de Fijter[2], Brendan J. Keating[4], Andreas Heinzel[5], Roman Reindl-Schwaighofer[5], Rainer Oberbauer[5], Frans H.J. Claas[1], Michael Eikmans[1]

[1] Dept. of Immunohematology and Blood Transfusion, [2] Dept. of Nephrology, Leiden University Medical Center, Leiden, the Netherlands.
[3] Dept. of Genetics, University Medical Center Utrecht, Utrecht, the Netherlands.
[4] Dept. of Pediatrics, University of Pennsylvania, Philadelphia, PA, USA.
[5] Department of Internal Medicine III, Medical University of Vienna, Vienna, Austria.

Abstract

Background Biopsy-proven acute rejection (BPAR) is a risk factor for adverse kidney transplant outcome. Previous hypothesis-driven studies have led to identification of genetic variants associated with AR. Here, we conducted a genome-wide association study (GWAS) to search in an unbiased manner for loci and single nucleotide polymorphisms (SNPs) that are associated with BPAR after kidney transplantation.

Method A total of 325 patients and 321 donors, transplanted between 1994 and 2012, were genotyped on the Transplant SNP array v1. The genotyped dataset was imputed based on a combined reference set derived from the 1,000 Genomes Project and Genome of the Netherlands, resulting in seven million analysable SNPs after rigorous quality control. Genetic associations were tested by factored spectrally transformed linear mixed models (FaST-LMM). Identified candidate SNPs in the GWAS discovery cohort were tested in an independent cohort 243 recipients from another transplant center. A set of previously published candidate risk genes of allograft rejection was interrogated in the current GWAS.

Results After correction for clinical risk factors, candidate loci were identified in the patients: top ranking variant rs112775512 (OR=1.44, $P=3.38\times10^{-9}$) in an intron of *COL5A1*, which was associated with BPAR. None of the significant candidate SNPs ($P<5\times10^{-6}$) identified in the discovery cohort could be confirmed in the validation cohort. In previously published genetic association studies, we could confirm the association of rs1801274 in FCGR2 with BPAR.

Conclusion These findings emphasize the importance of validation in genetic association studies. International collaborative studies in the field of kidney transplantation are necessary to increase sample size and identify robust clinically relevant SNPs.

Introduction

Acute allograft rejection remains a risk factor for adverse kidney transplant outcome (1). Human leukocyte antigen (HLA) mismatching between donor and recipient, the presence of anti-HLA antibodies in the patient, delayed graft function (DGF), and younger patient age are considered as risk factors for acute rejection (AR) (2-4). Besides the well-defined clinical and immunological risk factors, genetic variants across the human genome may influence allograft rejection (5, 6). Genetic studies in renal transplant have mainly focused on single nucleotide polymorphisms (SNPs) located within or flanking the genes that encode for cytokines, chemokines, toll-like receptors, ficolins, and complement components, which play a role in immune responses (7-11). These studies have led to inconsistent results, probably due to limited sample size, different population substructures, and the lack of validation in an independent cohort (5, 11).

Identifying genetic variants that underlie allograft rejection is rather complex, since acute rejection is affected by the extent of alloreactivity of the patient's immune system toward the donor organ and the effect of the immunosuppression applied. The choice of candidate genes that have been studied in previous genetic studies has relied heavily on their relationship to the known pathophysiology of rejection, but such studies may not fully explain the genetic basic of allograft rejection (12). Genome-wide association studies (GWAS) represent a hypothesis free approach to identify causal genetic variants by analysing millions of SNPs scattered across genome. GWAS had been successfully applied for identification novel genes in many diseases, such as diabetes and Alzheimer's disease (13, 14). However, in the kidney transplantation field only a few studies have reported novel loci that are associated with acute rejection or long-term kidney function (15, 16). Multicenter GWAS in renal transplants led to identification of two loci, which constitute CCDC67 and PTPRO, associated with biopsy proven acute T cell mediated rejection in both a discovery and validation cohort (15). Another GWAS identified two SNPs (rs3811321 and rs6565887) associated with 5-year creatinine levels and long-term graft survival (16). However, in a larger follow-up study involving 1,638 patients the impact of those two SNPs on long-term graft function could not be confirmed (17). This indicates the importance of validation of GWAS studies and asks for multicenter collaboration in the field of transplantation to increase sample sizes.

In the present study, we applied GWAS to identify novel loci that are associated with the occurrence of biopsy proven acute rejection after kidney transplantation. We also interrogated previously reported SNPs and their possible association with renal transplant outcome in this cohort.

Materials and Methods

Patients and donors

Patients receiving a kidney transplant between 1994 and 2012 in the Leiden University Medical Center (LUMC) were investigated. Thirty-five out of 646 DNA samples were excluded due to poor quality of genotyping (QC call rate < 95%). Finally, there were 611 DNA samples, from 305 patients and 306 donors. Cases were defined as patients developing biopsy proven acute rejection (BPAR) based on Banff classification. Controls were defined as patients with stable graft function, having no indication of clinical rejection. Patients with an episode of BPAR after switching of maintenance medication or with only a clinical indication of rejection without biopsy were excluded (Figure 1).

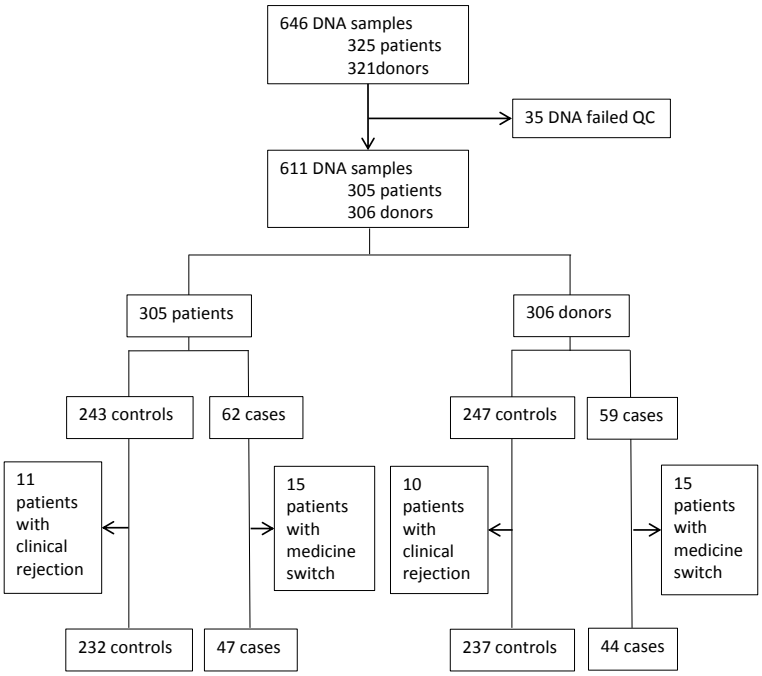


Figure 1. Flowchart of patients and donors included in discover cohort. QC: quality control.

Genotyping

Patient and donor DNA was isolated using chemagic DNA Blood2k Kit by chemagic MSM I equipment (PerkinElmer), and the quantity was measured on a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc, Asheville NC). Extracted DNA was diluted to 50 ng/µL with nuclease-free water. All DNA samples were genotyped by transplant SNP

array on the Affymetrix platform, which contain 753,182 SNPs, including fine-mapping SNPs across HLA region and drug-response associated loci (18).

Quality control and Imputation

Normal quality control was applied on raw genotype data to remove low quality samples and SNPs. Five samples with more than 5% missing genotypes were excluded. Individual SNPs with a Hardy-Weinberg equilibrium (HWE) P-value of less than 10^{-3}, monomorphic SNPs, and SNPs with a genotype call rate lower than 95% were excluded. The final dataset contained 611 samples and 592,990 SNPs for imputation.

Imputation was performed using IMPUTE2 based on a combined reference set (1,000 Genomes Project and Genome of The Netherlands, GoNL) (19). A post-imputation quality control was applied to filter out SNPs that did not meet our criteria (info-score < 0.7, HWE p-value < 10^{-3}, SNP call rate < 0.95, MAF < 0.05), which result in a total of 7,067,718 SNPs that were analyzed for association. All the imputed genotyped data were transformed into PLINK format using PLINK software, version 1.9.

Statistical analysis

Associations between clinical phenotypes (biopsy proven acute rejection) and genotypes were tested using factored spectrally transformed linear mixed models (FaST-LMM) to capture population structure, family structure, and cryptic relatedness (20). Clinical risk factors with a P-value less than 0.1 were included in the linear mixed model. The covariant included different therapeutic regimes, patient age and gender, donor type at transplant (living or deceased), and CMV primo-infection. Quantile-quantile (Q-Q) and Manhattan plots were generated using the qqman package (21). Genome-wide distribution of the test statistics showed no systematic inflation by inspecting the Q-Q plot and calculating the genomic inflation factor (λ). Suggestive association threshold ($P<5\times10^{-6}$) and genome-wide significance threshold ($P<5\times10^{-8}$) were used as correction for multiple testing.

Power calculation

The power of the GWAS, demonstrated as the relative risk versus minor allele frequency, was calculated by PGA software (22). We calculated the power using a disease prevalence of 0.15 and statistic power of 0.8, with the threshold of significance $P=5\times10^{-6}$ by default model.

Validation of candidate SNPs associated with kidney transplant outcome

A total of 67 previous published SNPs, located in genes encoding cytokines, chemokines, and innate immune response molecules (reviewed in (23)), were associated with acute rejection after kidney transplantation in the patients. For polymorphisms in the donor, 11 SNPs were reported to be significant association with allograft rejection. These candidate SNPs were validated in the present GWAS in association with BPAR. A P value of less than 0.05 was considered as significant.

Results

Patient characteristics and outcomes

A discovery cohort of 305 patients and 306 donors was genotyped by Affymetrix transplant arrays and passed the quality control. The patient and donor factors (age and gender) were not different between controls and BPAR group. Patients in the studies received different therapeutic regimes, consisting of mycophenolate mofetil (MMF) and calcineurin inhibitor (CNI), after kidney transplantation, which was a risk factor for BPAR. A deceased donor graft and CMV primo infection were risk factors for BPAR, whereas occurrence of DGF, HLA mismatching between donor and recipient, and younger recipient age did not predict occurrence of acute rejection (Table 1).

Table 1. Demographics of study cohort [1]

Variables	Controls (N=232)	Cases (N=47)	P [2]
Recipient age (IQR, year)	54.0 (43.3-60.0)	50.0 (37.0-63.0)	0.501
Recipient gender (female)	33.6%	23.4%	0.171
First transplant	95.7%	93.6%	0.539
Donor age (IQR, year)	49.0 (39.0-59.0)	51.0 (39.0-57.0)	0.893
Donor gender (female)	50.4%	59.6%	0.253
Donor type (deceased)	55.6%	72.3%	0.034*
Cold ischemia time (IQR, h)	17.3 (13.4-20.2) [a]	17.4 (12.8- 22.4) [b]	0.631
DGF (within deceased donor)	61.2% [c]	73.5% [d]	0.185
HLA-A matching	28.5%	36.2%	0.421
HLA-B matching	17. 7%	23.4%	0.322
HLA-DR matching	28.9%	19.2%	0.345
HLA-DQ matching	46.1%	40.4%	0.690
CMV primo-infection	3.9%	10.6%	0.067
Maintenance therapy			0.003*
CNI, MMF	6.5%	12.8%	
Tac, MMF	13.4%	8.5%	
CsA, MMF	43.5%	66.0%	
CNI, MMF, steroid	36.6%	12.8%	

[1] HLA, human leukocyte antigen; DGF, delayed graft function; CMV, Cytomegalovirus; MMF, mycophenolate mofetil; CsA, cyclosporine A; Tac, Tacrolimus; CNI, calcineurin inhibitor.

[2] P-values were calculated using the Mann-Whitney test, Chi-square test or Fisher exact test.

* indicates significance.

[a, b, c, d] data missing for 136, 10, 103, 13 patients

GWAS revealed three loci for BPAR in the recipient

All SNPs passing the QC were tested using the linear mixed model, which was adjusted for clinical risk factors and genetic background. The Q-Q plot showed an effective control of population structure (λ=1.07). The tail of Q-Q curve deviating from the expected distribution may indicate true association (Figure S1). Nine SNPs showed association with BPAR with a $P<5\times10^{-8}$, as shown in a Manhattan Plot (Figure 2). Seven of the nine SNPs identified (top-ranked SNP: rs112775512) are located in the intron of collagen type V alpha 1 (COL5A1). One SNP (rs3057090) is located 20 kb downstream of Dishevelled associated activator of morphogenesis 1 (DAAM1) and one SNP (rs77493583) is located in the intron of DPY19L1.

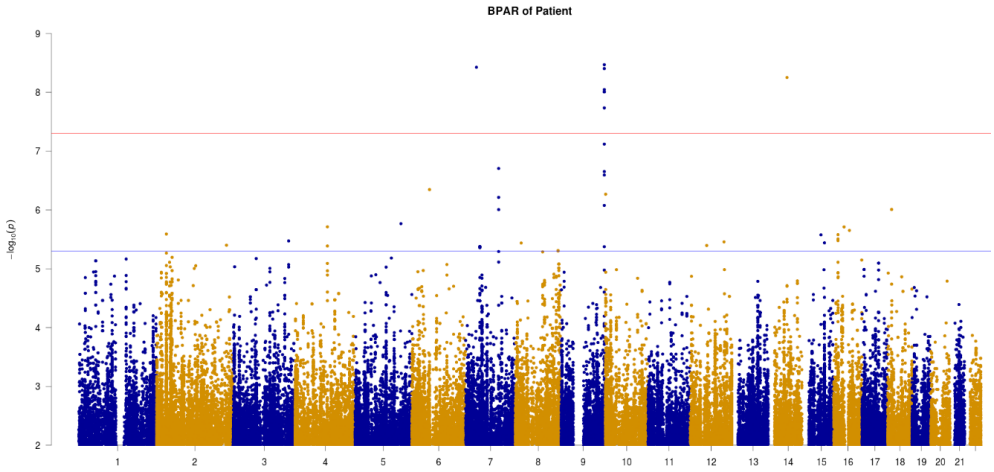


Figure 2. Manhattan plot of SNPs associated with BPAR in patients. Overall, 7 million SNPs were analyzed in relation to BPAR, with correction of clinical risk factors. The figure shows p-values (P<0.01) of each SNP against the chromosomal positions. The red line shows the threshold of genome wide significance ($P<5\times10^{-8}$) and the blue line indicates the genome wide suggestive significance ($P<5\times10^{-6}$).

Genetic variants validation in independent cohort

The validation cohort contained 243 recipients from the transplantation center in Vienna, which had been genotyped using the same Transplant arrays. A total of 44 candidate genetic variants ($P<5\times10^{-6}$) were eligible for association analysis (Table 2). Association of candidate SNPs were tested by the FaST-LMM algorithm, correcting for any significant clinical risk factors. Three SNPs showed significant association with BPAR (P<0.05), but the odds ratio were in opposite direction compared to those in the discovery cohort.

Table 2. Associations of genetic loci with BPAR in two cohorts [1].

SNP			Discovery cohort		Validation cohort	
Chr	SNP ID	Minor allele	P [2]	OR	P [2]	OR
2	rs62178588	G	3.97E-06	0.85	0.012*	1.14
2	rs74765547	TGACTGCTGAAAACAC	2.56E-06	1.24	0.582	0.97
3	rs7627382	T	3.37E-06	1.24	0.106	0.91
4	rs34790532	T	4.10E-06	1.24	0.046*	0.89
4	rs72684896	T	1.93E-06	1.25	0.041*	0.89
5	rs2116800	A	1.71E-06	1.21	0.909	1.01
6	rs76468144	T	4.50E-07	1.48	0.628	0.95
7	rs1826839	A	4.37E-06	1.17	0.301	1.05
7	rs1826840	A	4.37E-06	1.17	0.256	1.06
7	rs2331387	A	4.20E-06	1.17	0.372	1.04
7	rs2331389	T	4.20E-06	1.17	0.372	1.04
7	rs2526975	A	1.97E-07	0.85	0.694	1.02
7	rs2727762	T	6.10E-07	1.18	0.433	1.04
7	rs396600	C	9.86E-07	1.19	0.505	1.03
7	rs4724442	A	4.20E-06	1.17	0.276	1.05
7	rs77493583	C	3.75E-09	1.44	0.67	0.96
7	rs9690070	C	4.20E-06	1.17	0.372	1.04
8	rs1873654	T	3.64E-06	1.28	0.259	1.11
8	rs4909457	T	4.90E-06	1.16	0.923	1
8	rs7386038	T	4.98E-06	1.16	0.927	1
9	rs11103457	T	8.37E-07	1.22	0.886	1.01
9	rs112775512	A	3.39E-09	1.44	0.216	0.9
9	rs118029018	C	9.82E-09	1.42	0.193	0.89
9	rs12001485	A	9.82E-09	1.42	0.284	0.91
9	rs143702384	CA	3.95E-09	1.44	0.18	0.89
9	rs145688704	T	4.22E-06	1.2	0.893	1.01
9	rs372298474	G	2.22E-07	1.36	0.491	0.95
9	rs66698367	T	7.60E-08	1.36	0.223	0.9
9	rs67349136	G	1.84E-08	1.41	0.352	0.93
9	rs72772536	T	9.82E-09	1.42	0.444	0.94
9	rs72772543	C	9.00E-09	1.42	0.573	0.95
9	rs72772548	T	2.55E-07	1.4	0.395	0.92
10	rs35772020	A	5.40E-07	1.43	0.724	0.97
12	rs75575129	A	3.48E-06	1.39	0.831	0.98
12	rs79634630	A	4.01E-06	1.47	0.558	1.09
14	rs3057090	CTTGTTG	5.60E-09	1.44	0.316	1.09

15	rs11632600	T	2.65E-06	1.26	0.473	0.96
15	rs71395028	A	3.62E-06	1.37	0.31	1.13
16	16:32534119	C	1.94E-06	1.57	0.707	0.95
16	rs112139404	AC	2.22E-06	1.43	0.843	0.98
16	rs1230896	T	2.63E-06	1.18	0.666	1.02
16	rs251919	T	3.32E-06	1.18	0.658	1.02
16	rs436054	C	3.11E-06	1.18	0.62	1.02
18	rs67127738	A	9.81E-07	1.2	0.633	0.97

[1] SNP, single nucleotide polymorphism; Chr, chromosome; OR, odds ratio.

[2] P-values were calculated using FaST-LMM algorithm.

Donor GWAS for BPAR

We also performed a GWAS to study the donor genotype in relation to BPAR. The Q-Q plot showed appropriate control of population substructure, but no evidence of association with BPAR (Figure S2). Only one SNP (rs79712820) was significantly ($P<5\times10^{-8}$) associated with BPAR (Figure 3). This SNP was located in the intron of synaptopodin-2, which has actin binding and bundling activity.

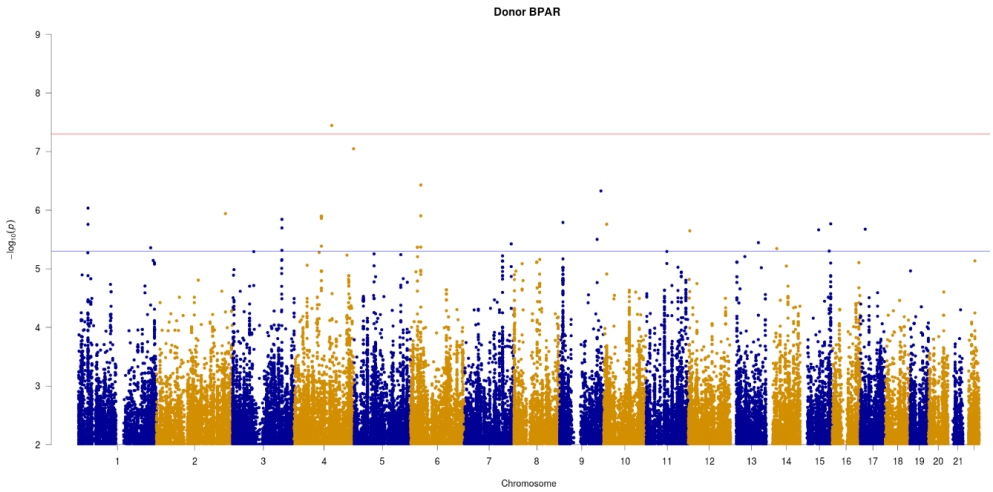


Figure 3. Manhattan plot of SNPs associated with BPAR in donors. The figure shows p-values ($P<0.01$) of each SNP against the chromosomal positions. The red line shows the threshold of genome wide significance ($P<5\times10^{-8}$) and the blue line indicates the genome wide suggestive significance ($P<5\times10^{-6}$).

Validation of previous published candidate SNPs in the current GWAS

We attempted to validate previous publications, which reported 67 SNPs in genes encoding for cytokines, chemokines, cell adhesion molecules, and innate immunity related molecules (23). In total, 59 SNPs were successfully captured in our patient GWAS dataset. Only three of them were significantly associated with BPAR (Table 3). Whereas two SNPs (rs2515641 and rs5742909) showed an opposite effect on AR compared with previous publications. One SNP (rs1801274), located in FCGR2A, was associated with BPAR (P<0.05).

Table 3. Validation of candidate SNPs that were reported in previous genetic association studies of transplant outcome [1]

SNP			Previous publication			Current GWAS		Ref
Chr	SNP ID	Gene	N	P	OR (95% CI)	P [3]	OR	
1	rs2796267	CD46	334	0.012	0.47 (0.26-0.84)	0.75	0.99	(27)
1	rs1801274	FCGR2A	99	<0.045	AR: more C allele [2]	0.002*	0.91	(28)
1	rs1800896	IL10	291	0.016	1.9 (1.1-3.1)	0.06	1.06	(7)
1	rs1800871	IL10	291	0.016	1.9 (1.1-3.1)	0.75	0.99	(7)
1	rs1800872	IL10	291	0.016	1.9 (1.1-3.1)	0.75	0.99	(7)
1	rs1801133	MTHFR	585	0.012	0.51 (0.3-0.86)	0.50	1.02	(29)
1	rs689466	PTGS2	458	0.01	0.59 (0.38-0.91)	0.44	1.03	(30)
2	rs3116496	CD28	270	0.026	1.93 (1.10-3.39)	0.87	0.99	(31)
2	rs733618	CTLA4	167	0.002	0.41 (0.24-0.72)	0.10	1.09	(32)
2	rs5742909	CTLA4	131	0.015	3.45 (1.18-10.1)	0.01*	0.84	(33)
2	rs231775	CTLA4	190	0.037	2.78 (1.07-7.19)	0.06	1.06	(34)
2	rs3087243	CTLA4	72	0.035	4.51	0.47	0.98	(35)
2	rs1143634	IL1B	100	0.045	3.11 (1.02-9.44)	0.10	0.94	(36)
2	rs7574865	STAT4	453	0.003	0.54 (0.36-0.82)	0.71	0.99	(37)
2	rs17868320	UGT1A9	100	0.07	3.62 (0.90-14.5)	0.71	0.97	(38)
2	rs6714486	UGT1A9	100	0.05	4.40 (1.05-18.4)	0.43	0.94	(38)
3	rs5186	AT1R	206	<0.05	8.34 (2.43-28.69)	0.24	0.96	(39)
3	rs1799864	CCR2	163	0.014	0.30 (0.12-0.78)	0.41	1.05	(40)
3	rs1799987	CCR5	243	0.029	2.76 (1.11-6.90)	0.07	0.95	(41)
3	rs1129055	CD86	Meta	0.02	0.35 (0.14-0.85)	0.46	1.03	(42)
3	rs11706052	IMPDH2	232	0.006	3.39 (1.42-8.09)	0.74	0.98	(10)
4	rs4073	CXCL8	296	0.032	2.7 (1.09-6.69)	0.90	1.00	(43)
4	rs2069762	IL2	63	<0.05	NA	0.69	1.01	(44)
4	rs28362491	NFKB1	292	0.001	2.61 (1.50-4.53)	0.10	1.05	(45)
4	rs7439366	UGT2B7	235	<0.046	2.5 (1.00-6.41)	0.05	0.94	(38)
5	rs181781	IL3	330	0.041	0.55 (0.31-0.98)	0.33	0.95	(46)
5	rs40401	IL3	330	0.014	2.18 (1.17-4.05)	0.09	0.94	(46)

5	rs2243250	IL4	120	0.02	NA	0.83	0.99	(47)
5	rs2910164	MIR146A	350	0.04	2.63 (1.04-6.62)	0.94	1.00	(48)
6	rs2269475	AIF	458	0.05	0.61 (0.39-0.97)	0.29	0.95	(30)
6	rs1800629	TNF	623	0.001	4.05 (1.76-9.28)	0.17	0.95	(49)
6	rs699947	VEGFA	173	0.005	4.1 (1.5-11.3)	0.41	1.02	(50)
6	rs1570360	VEGFA	173	0.001	6.8 (1.8-25.0)	0.93	1.00	(50)
7	rs2032582	ABCB1	232	0.003	3.16 (1.50-6.67)	0.26	0.96	(10)
7	rs1800795	IL6	145	0.0002	NA	0.15	0.96	(51)
7	rs2278293	IMPDH1	191	0.008	0.34 (0.15-0.76)	0.32	0.97	(52)
7	rs2278294	IMPDH1	191	0.02	0.40 (0.18-0.89)	0.62	0.98	(52)
8	rs1042032	EPHX2	259	0.015	6.34 (1.35-29.9)	0.62	0.98	(53)
9	rs4986790	TLR4	238	0.01	0.41 (0.30-0.83)	0.82	1.01	(54)
9	rs10759932	TLR4	216	0.001	0.25 (0.11-0.57)	0.77	0.99	(55)
10	rs2515641	CYP2E1	347	0.003	2.55 (1.37-4.75)	0.04*	0.91	(56)
10	rs7096206	MBL2	710	0.01	2.05 (1.16-3.64)	0.86	0.99	(57)
11	rs10765602	CCDC67	778	0.02	1.98 (1.21-3.25)	0.94	1.00	(15)
11	rs187238	IL18	226	0.015	3.65 (1.24-10.79)	0.25	1.04	(58)
12	rs2430561	INFG	118	NA	2.6 (1.6-6.0)	0.48	0.97	(59)
12	rs7976329	PTPRO	778	0.01	1.61 (0.96-2.70)	0.09	0.94	(15)
12	rs11614913	MIR196A2	350	0.027	2.86 (1.12-7.27)	0.68	1.01	(48)
14	rs696	NFKBIA	292	0.007	1.85 (1.18-2.91)	0.79	0.99	(45)
16	rs1801275	IL4R	344	0.019	0.44 (0.21-0.91)	0.11	0.94	(60)
17	rs1024611	CCL2	167	0.022	2.6 (1.12-6.01)	0.57	0.98	(61)
17	rs2107538	CCL5	261	0.035	NA	0.92	1.00	(62)
17	rs5918	ITGB3	119	0.04	2.75 (1.01-7.93)	0.95	1.00	(63)
17	rs1625895	TP53	100	0.009	0.36 (0.16-0.78)	0.25	0.95	(64)
19	rs5498	ICAM1	42	0.013	0.23	0.97	1.00	(65)
19	rs1800470	TGFB	291	0.043	1.8 (1.0–3.0)	0.19	0.96	(7)
19	rs1800471	TGFB	291	0.043	1.8 (1.0-3.0)	0.53	1.04	(7)
20	rs3746444	MIR499A	350	0.027	3.31 (1.14-9.58)	0.37	0.97	(48)
22	rs228942	IL2RB	337	0.0096	2.11 (1.19-3.74)	0.45	1.03	(66)
22	rs228953	IL2RB	337	0.029	1.58 (1.04-2.38)	0.95	1.00	(66)

[1] SNP, single nucleotide polymorphism; Chr, chromosome; OR, odds ratio; NA, not available; CI, Confidence interval; Ref, reference.

[2] OR was not given in study.

[3] P-values in current GWAS were calculated using FaST-LMM algorithm.

From our donor GWAS dataset, a total of 11 previously published SNPs associated with allograft rejection could be successfully captured (Table 4). However, none of these variants was significantly associated with BPAR in the current study.

Table 4. Validation of candidate SNPs that were reported in previous genetic association studies of the transplant donor with outcome [1]

SNP			Previous publications			Current GWAS		Ref
Chr	SNP ID	Gene	N	P	OR (95% CI)	P [2]	OR	
3	rs1799987	CCR5	239	0.029	NA	0.61	1.02	(67)
6	rs1570360	VEGF	173	0.001	2.2 (1.4-3.7)	0.60	1.02	(50)
6	rs1800629	TNFa	120	0.0395	1.4	0.36	1.04	(68)
6	rs699947	VEGF	173	0.005	1.9 (1.2-3.0)	0.66	1.01	(50)
7	rs2069840	IL-6	145	0.0002	8.67	0.27	0.96	(51)
8	rs1042032	EPHX2	259	0.042	5.53 (1.10–27.80)	0.79	0.99	(53)
9	rs4986790	TLR4	122	0.02	NA	0.96	1.00	(69)
9	rs4986791	TLR4	122	0.02	NA	0.51	1.04	(69)
9	rs7851696	Ficolin-2	270	0.048	1.71 (1.02-2.87)	0.66	0.98	(70)
10	rs1800682	FAS	105	0.043	3.27 (1.04-10.32)	0.64	1.01	(71)
10	rs1801157	SDF1	335	0.006	0.39 (0.21–0.76)	0.34	1.04	(72)

[1] SNP, single nucleotide polymorphism; Chr, chromosome; OR, odds ratio; NA, not available; CI, Confidence interval; Ref, reference.

[2] P-values in current GWAS were calculated using FaST-LMM algorithm.

Discussion

We performed a GWAS to investigate genetic variants associated with biopsy proven acute rejection (BPAR) in kidney transplantation. Several SNPs were identified in our discovery GWAS cohort to be associated to BPAR, but unfortunately none of these could be verified in an independent cohort. We could confirm in our cohort the association of rs1801274 in FCGR2 with BPAR, which has been described in a previous genetic association study.

In the transplant recipients, nine SNPs reached genome wide significant level, of which seven are located in the intron of COL5A1 at chromosome 9, and two are lone SNPs located on chromosome 7 and 14. These multiple continuous SNPs were more likely assumed to be true risk variants for BPAR than the lone SNP because of linkage disequilibrium. Although the two lone SNPs were significantly associated with BPAR with high imputation certainty (info>0.9), this finding requires further replication. In an independent validation cohort, the nine candidate SNPs showed no significant association with BPAR. Also, the SNPs

showing association to BPAR with a suggestive P value ($P<5\times10^{6}$) could not be verified in the replication cohort.

Although one SNP reached the significant level in donor GWAS, it did not show large deviation from expected distribution in the Q-Q plot. In addition, the relative small risk effect suggests that this SNP was found by chance. These results suggest that donor polymorphisms confer no big effect on the risk of acute rejection.

The main reason, that findings in the discovery cohort could not be verified in the validation cohort, may be the relatively small sample size and limited power to detect any small effects on outcome of single SNPs. Power calculation showed that SNPs (MAF=0.2) with a relative risk of more than 2.8 could be sufficiently detected (power=0.8) at the genome wide suggestive significance level (Figure 4). However, our results showed that the relative risk calculated by linear mixed model is less than two, indicating that the candidate SNPs may have been identified by chance. In other words, our study is only able to sufficiently detect SNPs (MAF>0.2) that have a relatively big effect on BPAR (RR>2.8). The validation cohort with comparable sample size had modest power to detect variants at the conventional level (P<0.05).

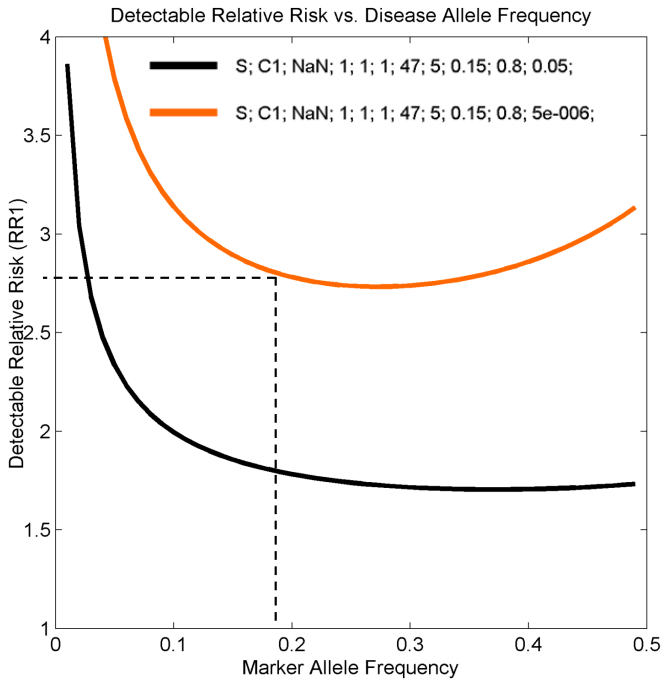


Figure 4. Power of the study. The plot shows relative risk versus minor allele frequency. The power was calculated using a disease prevalence of 0.15 and a statistic power of 0.8 by default model. The orange line and black line show the threshold of suggestive significance ($P=5\times10^{-6}$) and the unadjusted significance (P=0.05), respectively.

Apart from any novel loci, risk loci that have previously been discovered in genetic risk studies were also evaluated in the current GWAS. Variant rs7976329, located in the intron of *PTPRO* and rs10765602 located in the upstream of *CCDC67*, confer a modest effect on BPAR (RR<2) (15). Our study had sufficient power to detect these variants at the conventional significance level (P<0.05). However, these two risk variants showed no association with acute rejection in our study cohort, which suggests that variation between transplant centers may hamper validation in genomic studies. Similarly, two variants (rs3811321 and rs6565887) associated with long-term kidney function in 326 renal transplant recipients could not be confirmed in an independent study on 1,638 patients (16, 17).

Failed validation is quite common in clinical studies especially in the field of transplantation. A well-powered GWAS in blood or marrow transplantation (BMT) demonstrated that a substantial amount of candidate SNPs identified by previous publications showed falsely positive association with survival outcomes (24). Similarly, our current GWAS failed to validate 69 out of 70 genetic variants previously reported to be associated with acute rejection. Only rs1801274 (FCGR2A) could be confirmed at the conventional significance level, whereby the recipients with acute rejection in both studies have higher C allele frequency. Our results highlight the importance of validating SNPs identified by candidate approaches or unbiased genome wide associations, and they demand large collaborative studies of the genetic effect on kidney transplant outcome.

As Stegall and colleagues have mentioned, complex outcomes such as graft loss or acute rejection may have numerous causes, and are unlikely to be fully related to variants in one gene (25). BPAR is associated with the donor organ quality, HLA matching status, and immunosuppressive therapy. Therefore, any effect of genetic variants on acute rejection may be counterbalanced by other potential factors, and thus they may be hard to capture in genetic association studies. At the other hand, the less complex the outcome, the more likely it is to discover robust gene associations. For example, the DeKAF consortium identified by GWAS in African American kidney transplant recipients two additional CYP3A5 alleles that are associated with tacrolimus trough blood concentrations (26). Indeed, the association between pharmacogenetic polymorphisms and tacrolimus concentrations has been widely validated in many studies (23).

In conclusion, we identified several SNPs in the patient and the donor by GWAS, which were associated with occurrence of acute rejection. These could unfortunately not be confirmed in an independent cohort. International collaborative studies are highly recommended to obtain adequate power and overcome any falsely positive findings. Of the SNPs that have previously been described to predict transplant outcome, we could confirm association of rs1801274 in FCGR2 with occurrence of acute rejection. Further studies are needed to establish the function of this molecule in transplantation.

References

1. Mateu LMP, Calabuig AS, Plaza LC, Esteve AF. Acute rejection and late renal transplant failure: risk factors and prognosis. Nephrology Dialysis Transplantation. 2004;19(suppl 3):iii38-iii42.
2. Wissing KM, Fomegné G, Broeders N, Ghisdal L, Hoang AD, Mikhalski D, et al. HLA mismatches remain risk factors for acute kidney allograft rejection in patients receiving quadruple immunosuppression with anti-interleukin-2 receptor antibodies. Transplantation. 2008;85(3):411-6.
3. Lebranchu Y, Baan C, Biancone L, Legendre C, Morales JM, Naesens M, et al. Pretransplant identification of acute rejection risk following kidney transplantation. Transplant International. 2014;27(2):129-38.
4. Wu WK, Famure O, Li Y, Kim SJ. Delayed graft function and the risk of acute rejection in the modern era of kidney transplantation. Kidney international. 2015;88(4):851-8.
5. Goldfarb-Rumyantzev AS, Naiman N. Genetic predictors of acute renal transplant rejection. Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association. 2010;25(4):1039-47.
6. Tran T, Unterrainer C, Fiedler G, Döhler B, Scherer S, Ruhenstroth A, et al. No Impact of KIR-Ligand Mismatch on Allograft Outcome in HLA-Compatible Kidney Transplantation. American Journal of Transplantation. 2013;13(4):1063-8.
7. Alakulppi NS, Kyllonen LE, Jantti VT, Matinlauri IH, Partanen J, Salmela KT, et al. Cytokine gene polymorphisms and risks of acute rejection and delayed graft function after kidney transplantation. Transplantation. 2004;78(10):1422-8.
8. Brown KM, Kondeatis E, Vaughan RW, Kon SP, Farmer CK, Taylor JD, et al. Influence of donor C3 allotype on late renal-transplantation outcome. The New England journal of medicine. 2006;354(19):2014-23.
9. Krüger B, Krick S, Dhillon N, Lerner SM, Ames S, Bromberg JS, et al. Donor Toll-like receptor 4 contributes to ischemia and reperfusion injury following human kidney transplantation. Proceedings of the National Academy of Sciences. 2009;106(9):3390-5.
10. Grinyo J, Vanrenterghem Y, Nashan B, Vincenti F, Ekberg H, Lindpaintner K, et al. Association of four DNA polymorphisms with acute rejection after kidney transplantation. Transplant international : official journal of the European Society for Organ Transplantation. 2008;21(9):879-91.
11. Almoguera B, Shaked A, Keating BJ. Transplantation genetics: current status and prospects. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons. 2014;14(4):764-78.
12. Hirschhorn JN, Daly MJ. Genome-wide association studies for common diseases and complex traits. Nat Rev Genet. 2005;6(2):95-108.
13. McCarthy MI, Zeggini E. Genome-wide association studies in type 2 diabetes. Current diabetes reports. 2009;9(2):164-71.
14. Bertram L, Tanzi RE. Genome-wide association studies in Alzheimer's disease. Hum Mol Genet. 2009;18(R2):R137-45.
15. Ghisdal L, Baron C, Lebranchu Y, Viklický O, Konarikova A, Naesens M, et al. Genome-Wide Association Study of Acute Renal Graft Rejection. American Journal of Transplantation. 2017;17(1):201-9.
16. O'brien RP, Phelan PJ, Conroy J, O'kelly P, Green A, Keogan M, et al. A genome-wide association study of recipient genotype and medium-term kidney allograft function. Clinical transplantation. 2013;27(3):379-87.
17. Pihlstrøm HK, Mjøen G, Mucha S, Haraldsen G, Franke A, Jardine A, et al. Single Nucleotide Polymorphisms and Long-Term Clinical Outcome in Renal Transplant Patients: A Validation Study. American Journal of Transplantation. 2017;17(2):528-33.
18. Li YR, van Setten J, Verma SS, Lu Y, Holmes MV, Gao H, et al. Concept and design of a genome-wide association genotyping array tailored for transplantation-specific studies. Genome medicine. 2015;7(1):90.
19. Deelen P, Menelaou A, van Leeuwen EM, Kanterakis A, van Dijk F, Medina-Gomez C, et al. Improved imputation quality of low-frequency and rare variants in European samples using the 'Genome of The Netherlands'. European Journal of Human Genetics. 2014;22(11):1321-6.
20. Lippert C, Listgarten J, Liu Y, Kadie CM, Davidson RI, Heckerman D. FaST linear mixed models for genome-wide association studies. Nature methods. 2011;8(10):833-5.
21. Turner SD. qqman: an R package for visualizing GWAS results using QQ and manhattan plots. bioRxiv. 2014:005165.
22. Menashe I, Rosenberg PS, Chen BE. PGA: power calculator for case-control genetic association analyses.

BMC genetics. 2008;9(1):36.
23. Dorr CR, Oetting WS, Jacobson PA, Israni AK. Genetics of Acute Rejection after Kidney Transplantation. Transplant International. 2017.
24. Karaesmen E, Rizvi AA, Preus L, McCarthy PL, Pasquini MC, Onel K, et al. Replication and validation of genetic polymorphisms associated with survival after allogeneic blood or marrow transplant. Blood. 2017:blood-2017-05-784637.
25. Stegall MD, Park WD, Dierkhising R. Genes and transplant outcomes: the search for "associations". Transplantation. 2014;98(3):257-8.
26. Oetting W, Schladt D, Guan W, Miller M, Remmel R, Dorr C, et al. Genomewide association study of tacrolimus concentrations in African American kidney transplant recipients identifies multiple CYP3A5 alleles. American Journal of Transplantation. 2016;16(2):574-82.
27. Park M, Kim S, Lee T, Lee S, Moon J, Ihm C, et al., editors. A promoter polymorphism in the CD46 complement regulatory protein gene is associated with acute renal allograft rejection. Transplantation proceedings; 2016: Elsevier.
28. Yuan FF, Watson N, Sullivan JS, Biffin S, Moses J, Geczy AF, et al. Association of Fc gamma receptor IIA polymorphisms with acute renal-allograft rejection. Transplantation. 2004;78(5):766-9.
29. Oetting WS, Zhu Y, Brott MJ, Matas AJ, Cordner GK, Pan W. Validation of genetic variants associated with early acute rejection in kidney allograft transplantation. Clinical transplantation. 2012;26(3):418-23.
30. Vu D, Tellez-Corrales E, Shah T, Hutchinson I, Min DI. Influence of Cyclooxygenase-2 (COX-2) gene promoter-1195 and allograft inflammatory factor-1 (AIF-1) polymorphisms on allograft outcome in Hispanic kidney transplant recipients. Human immunology. 2013;74(10):1386-91.
31. Pawlik A, Dabrowska-Zamojcin E, Dziedziejko V, Safranow K, Domanski L. Association between IVS3+ 17T/C CD28 gene polymorphism and the acute kidney allograft rejection. Transplant immunology. 2014;30(2-3):84-7.
32. Gao J-w, Guo Y-f, Fan Y, Qiu J-x, Bao E-d, Liu Y, et al. Polymorphisms in cytotoxic T lymphocyte associated antigen-4 influence the rate of acute rejection after renal transplantation in 167 Chinese recipients. Transplant immunology. 2012;26(4):207-11.
33. Ruhi Ç, Sallakçi N, Yeğin O, Süleymanlar G, Ersoy FF. The influence of CTLA-4 single nucleotide polymorphisms on acute kidney allograft rejection in Turkish patients. Clinical transplantation. 2015;29(7):612-8.
34. Misra MK, Kapoor R, Pandey SK, Sharma RK, Agrawal S. Association of CTLA-4 gene polymorphism with end-stage renal disease and renal allograft outcome. Journal of Interferon & Cytokine Research. 2014;34(3):148-61.
35. Canossi A, Aureli A, Delreno F, Iesari S, Cervelli C, Clemente K, et al., editors. Influence of cytotoxic T-lymphocyte antigen-4 polymorphisms on acute rejection onset of cadaveric renal transplants. Transplantation proceedings; 2013: Elsevier.
36. Manchanda PK, Mittal RD. Analysis of cytokine gene polymorphisms in recipient's matched with living donors on acute rejection after renal transplantation. Molecular and cellular biochemistry. 2008;311(1-2):57-65.
37. Yang H, Zhou Q, Chen Z, Chen W, Wang M, Chen J. Polymorphisms in STAT4 increase the risk of acute renal allograft rejection in the Chinese population. Transplant immunology. 2011;24(4):216-9.
38. Pazik J, Ołdak M, Lewandowski Z, Podgórska M, Sitarek E, Płoski R, et al., editors. Uridine diphosphate glucuronosyltransferase 2B7 variant p. His268Tyr as a predictor of kidney allograft early acute rejection. Transplantation proceedings; 2013: Elsevier.
39. Zhang G, Wang H, Wang F, Yu L, Yang X, Meng J, et al. Gene polymorphisms of the renin-angiotensin-aldosterone system and angiotensin II type 1-receptor activating antibodies in renal rejection. The Tohoku journal of experimental medicine. 2007;213(3):203-14.
40. Abdi R, Tran TB, Sahagun-Ruiz A, Murphy PM, Brenner BM, Milford EL, et al. Chemokine receptor polymorphism and risk of acute rejection in human renal transplantation. Journal of the American Society of Nephrology : JASN. 2002;13(3):754-8.
41. Cha RH, Yang SH, Kim HS, Kim SM, Park MH, Ha J, et al. Genetic interactions between the donor and the recipient for susceptibility to acute rejection in kidney transplantation: polymorphisms of CCR5. Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association. 2009;24(9):2919-25.
42. Han FF, Fan H, Wang ZH, Li GR, Lv YL, Gong LL, et al. Association between co-stimulatory molecule gene

polymorphism and acute rejection of allograft. Transpl Immunol. 2014;31(2):81-6.
43. Singh R, Kesarwani P, Ahirwar DK, Kapoor R, Mittal RD. Interleukin 8 -251T>A and Interferon gamma +874A>T polymorphism: potential predictors of allograft outcome in renal transplant recipients from north India. Transpl Immunol. 2009;21(1):13-7.
44. Morgun A, Shulzhenko N, Rampim GF, Medina JO, Machado PG, Diniz RV, et al. Interleukin-2 gene polymorphism is associated with renal but not cardiac transplant outcome. Transplantation proceedings. 2003;35(4):1344-5.
45. Misra MK, Mishra A, Pandey SK, Kapoor R, Sharma RK, Agrawal S. Association of functional genetic variants of transcription factor Forkhead Box P3 and Nuclear Factor-kappaB with end-stage renal disease and renal allograft outcome. Gene. 2016;581(1):57-65.
46. Lee DY, Song SB, Moon JY, Jeong KH, Park SJ, Kim HJ, et al. Association between interleukin-3 gene polymorphism and acute rejection after kidney transplantation. Transplantation proceedings. 2010;42(10):4501-4.
47. Poole KL, Gibbs PJ, Evans PR, Sadek SA, Howell WM. Influence of patient and donor cytokine genotypes on renal allograft rejection: evidence from a single centre study. Transpl Immunol. 2001;8(4):259-65.
48. Misra MK, Pandey SK, Kapoor R, Sharma RK, Agrawal S. Genetic variants of MicroRNA-related genes in susceptibility and prognosis of end-stage renal disease and renal allograft outcome among north Indians. Pharmacogenetics and genomics. 2014;24(9):442-50.
49. Sanchez-Fructuoso AI, Perez-Flores I, Valero R, Moreno MA, Fernandez-Arquero M, Urcelay E, et al. The Polymorphism -308G/A of Tumor Necrosis Factor-alpha Gene Modulates the Effect of Immunosuppressive Treatment in First Kidney Transplant Subjects Who Suffer an Acute Rejection. 2016;2016:2197595.
50. Shahbazi M, Fryer AA, Pravica V, Brogan IJ, Ramsay HM, Hutchinson IV, et al. Vascular endothelial growth factor gene polymorphisms are associated with acute renal allograft rejection. Journal of the American Society of Nephrology. 2002;13(1):260-4.
51. Marshall SE, McLaren AJ, McKinney EF, Bird TG, Haldar NA, Bunce M, et al. Donor cytokine genotype influences the development of acute rejection after renal transplantation. Transplantation. 2001;71(3):469-76.
52. Wang J, Yang JW, Zeevi A, Webber SA, Girnita DM, Selby R, et al. IMPDH1 gene polymorphisms and association with acute rejection in renal transplant patients. Clinical pharmacology and therapeutics. 2008;83(5):711-7.
53. Gervasini G, García-Cerrada M, Coto E, Vergara E, García-Pino G, Alvarado R, et al. A 3'-UTR polymorphism in soluble epoxide hydrolase gene Is associated with acute rejection in renal transplant recipients. PloS one. 2015;10(7):e0133563.
54. Ducloux D, Deschamps M, Yannaraki M, Ferrand C, Bamoulid J, Saas P, et al. Relevance of Toll-like receptor-4 polymorphisms in renal transplantation. Kidney Int. 2005;67(6):2454-61.
55. Hwang YH, Ro H, Choi I, Kim H, Oh KH, Hwang JI, et al. Impact of polymorphisms of TLR4/CD14 and TLR3 on acute rejection in kidney transplantation. Transplantation. 2009;88(5):699-705.
56. Kim SK, Park HJ, Seok H, Jeon HS, Lee TW, Lee SH, et al. Association studies of cytochrome P450, family 2, subfamily E, polypeptide 1 (CYP2E1) gene polymorphisms with acute rejection in kidney transplantation recipients. Clin Transplant. 2014;28(6):707-12.
57. Golshayan D, Wojtowicz A, Bibert S, Pyndiah N, Manuel O, Binet I, et al. Polymorphisms in the lectin pathway of complement activation influence the incidence of acute rejection and graft outcome after kidney transplantation. Kidney Int. 2016;89(4):927-38.
58. Kim CD, Ryu HM, Choi JY, Choi HJ, Choi HJ, Cho JH, et al. Association of G-137C IL-18 promoter polymorphism with acute allograft rejection in renal transplant recipients. Transplantation. 2008;86(11):1610-4.
59. Tinckam KJ, Djurdjev O, Magil AB. Glomerular monocytes predict worse outcomes after acute renal allograft rejection independent of C4d status. Kidney international. 2005;68(4):1866-74.
60. Lee HJ, Kim TH, Kang SW, Kim YH, Kim SK, Chung JH, et al. Association Interleukin-4 and Interleukin-4 Receptor Gene Polymorphism and Acute Rejection and Graft Dysfunction After Kidney Transplantation. Transplantation proceedings. 2016;48(3):813-9.
61. Kang SW, Park SJ, Kim YW, Kim YH, Sohn HS, Yoon YC, et al. Association of MCP-1 and CCR2 polymorphisms with the risk of late acute rejection after renal transplantation in Korean patients. International journal of immunogenetics. 2008;35(1):25-31.

62. Kruger B, Boger CA, Obed A, Farkas S, Hoffmann U, Banas B, et al. RANTES/CCL5 polymorphisms as a risk factor for recurrent acute rejection. Clin Transplant. 2007;21(3):385-90.
63. Salido E, Martin B, Barrios Y, Linares JD, Hernandez D, Cobos M, et al. The PlA2 polymorphism of the platelet glycoprotein IIIA gene as a risk factor for acute renal allograft rejection. Journal of the American Society of Nephrology : JASN. 1999;10(12):2599-605.
64. Azarpira N, Kazemi K, Darai M. Influence of p53 (rs1625895) polymorphism in kidney transplant recipients. Saudi journal of kidney diseases and transplantation : an official publication of the Saudi Center for Organ Transplantation, Saudi Arabia. 2014;25(6):1160-5.
65. Tajik N, Salari F, Ghods AJ, Hajilooi M, Radjabzadeh MF, Mousavi T. Association between recipient ICAM-1 K469 allele and renal allograft acute rejection. International journal of immunogenetics. 2008;35(1):9-13.
66. Park SJ, Yoon YC, Kang SW, Kim TH, Kim YW, Joo H, et al. Impact of IL2 and IL2RB genetic polymorphisms in kidney transplantation. Transplantation proceedings. 2011;43(6):2383-7.
67. Hoffmann S, Park J, Jacobson LM, Muehrer RJ, Lorentzen D, Kleiner D, et al. Donor genomics influence graft events: the effect of donor polymorphisms on acute rejection and chronic allograft nephropathy. Kidney international. 2004;66(4):1686-93.
68. Lee H, Clark B, Gooi H, Stoves J, Newstead C. Influence of recipient and donor IL-1α, IL-4, and TNFα genotypes on the incidence of acute renal allograft rejection. Journal of clinical pathology. 2004;57(1):101-3.
69. Palmer SM, Burch LH, Mir S, Smith SR, Kuo PC, Herczyk WF, et al. Donor polymorphisms in Toll-like receptor-4 influence the development of rejection after renal transplantation. Clinical transplantation. 2006;20(1):30-6.
70. Dabrowska-Zamojcin E, Czerewaty M, Malinowski D, Tarnowski M, Słuczanowska-Głabowska S, Domanski L, et al. Ficolin-2 gene rs7851696 polymorphism is associated with delayed graft function and acute rejection in kidney allograft recipients. Archivum immunologiae et therapiae experimentalis. 2018;66(1):65-72.
71. Cappellesso S, Valentin JF, Giraudeau B, Boulanger MD, Al-Najjar A, Buchler M, et al. Association of donor TNFRSF6 (FAS) gene polymorphism with acute rejection in renal transplant patients: a case-control study. Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association. 2004;19(2):439-43.
72. Lee JP, Bae JB, Yang SH, Cha R-h, Seong EY, Park YJ, et al. Genetic predisposition of donors affects the allograft outcome in kidney transplantation; polymorphisms of stromal-derived factor-1 and CXC receptor 4. PloS one. 2011;6(2):e16710.

Supplementary Data

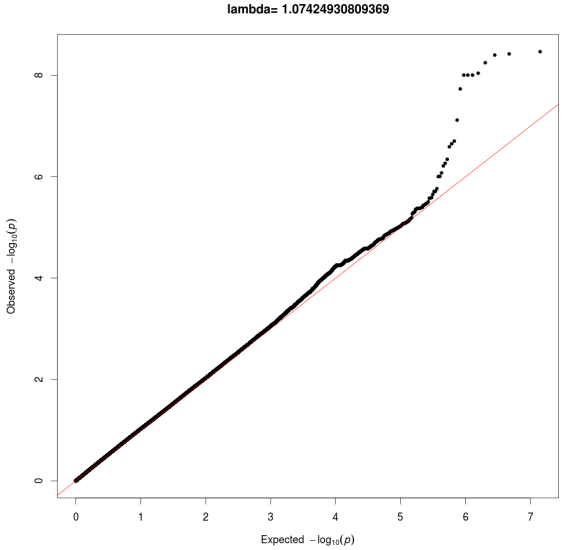


Figure S1. QQ plot for the patient's genetic variants in relation to BPAR. Figure showed an effective control of population structure (λ=1.07). The extreme observed P values may suggest association.

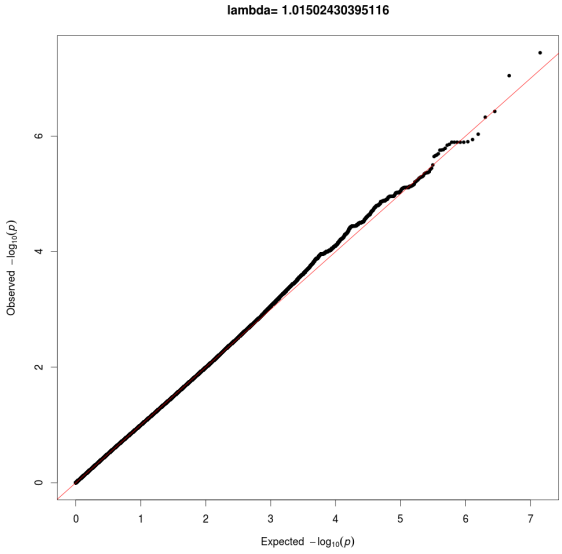
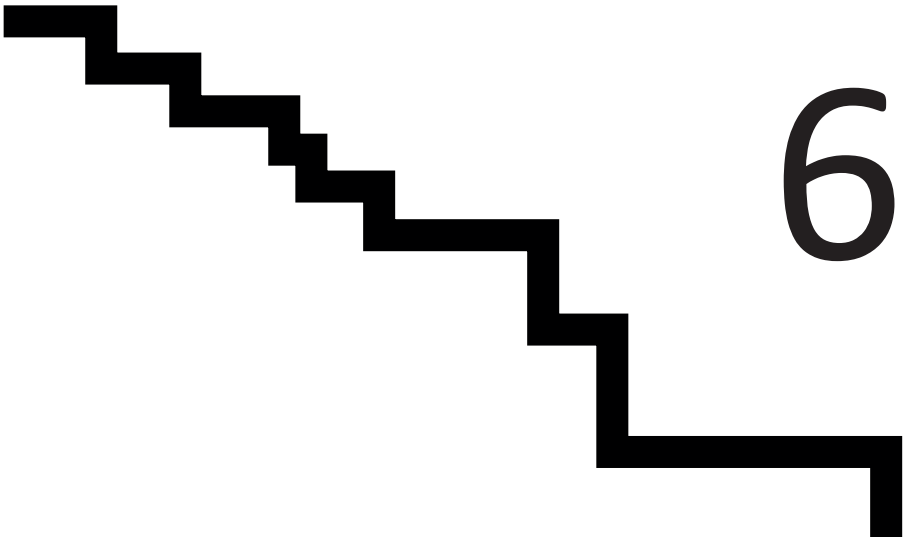


Figure S2. QQ plot for the donor's genetic variants in relation to BPAR. The figure shows the appropriate control of population structure (λ=1.02). The observed P values fall along a straight red line indicates that there is no true association with BPAR.

The degree of genomic missense SNP mismatching does not affect outcome after kidney transplantation



6

Jianxin Yang[1], Geertje J. Dreyer[2], Brendan J. Keating[3], Andreas Heinzel[4], Johan W. de Fijter[2], Frans H.J. Claas[1], Michael Eikmans[1]

[1]Dept. of Immunohematology and Blood Transfusion, [2] Dept. of Nephrology, Leiden University Medical Center, Leiden, the Netherlands.
[3]Dept. of Pediatrics, University of Pennsylvania, Philadelphia, PA, USA.
[4]Department of Internal Medicine III, Medical University of Vienna, Vienna, Austria.

Abstract

Background: Human leukocyte antigens (HLA) mismatching has an adverse effect on kidney transplant outcome. But even in fully HLA-compatible donor-recipient combinations graft loss occurs. We suspect that single nucleotide polymorphisms (SNPs) other than those in the HLA genes also play a role. Therefore, we investigated the effect of mismatching of genomic missense SNPs on occurrence of acute kidney allograft rejection and on two-year graft function.

Method: We performed a genome-wide association study to identify the association of mismatching of single missense polymorphisms between donor and recipient with transplant outcome. A total of 300 donor-recipient combinations were genotyped on the transplant SNP array for wide coverage of coding SNPs, insertion-deletion regions, loss of function variants. We pre-selected 10,800 missense SNPs (that cause an amino acid sequence alteration) and examined whether the mismatch of these SNPs is related to biopsy proven acute rejection (BPAR, either yes or no) and two-year estimated glomerular filtration rate (eGFR; either >40 mL/min or <=40 mL/min). Furthermore, a composite mismatch load score of all the missense SNPs was calculated and related to outcome.

Result: Mismatching of individual missense SNPs and the overall missense SNP mismatch load was not related to BPAR incidence and to graft function at two years.

Conclusion: The results suggested that missense SNPs mismatching does not have a strong effect on acute rejection and graft function in kidney transplantation.

Introduction

Acute allograft rejection remains a risk factor for adverse kidney transplant outcome (1, 2). Human leukocyte antigen (HLA) mismatching is considered as an immunological risk factor for acute rejection. Activated T cells against the incompatible donor HLA antigen secrete cytokines that drive an inflammatory cell infiltrate in the graft and that initiate cellular rejection (3, 4). Humoral alloimmune responses, characterized by the presence of donor specific HLA alloantibodies and complement activation, can lead to destruction of the donor organ (5, 6). Therefore, full HLA matching has a beneficial effect on long term graft survival, both after living and deceased donor transplantation (7, 8). However, allograft rejection and progressive graft loss do occur even in HLA matched transplants (9, 10).

The antigens responsible for rejection and graft loss in HLA identical transplantations are considered as minor histocompatibility antigens (miHA) (11, 12). The miHA are polymorphic peptides that typically arise from single-nucleotide polymorphisms (SNPs) and cause alterations in the amino acid sequence. Alloreactive T cells can recognize these miHA presented by the HLA molecules and subsequently initiate an immune response. Human H-Y antigen as miHA, a well-studied risk factor of graft-versus-host disease (GvHD) in hematopoietic stem-cell transplantation (HSCT), was associated with elevated risk of graft loss after kidney transplantation (9). Disparities between donor and recipient for HLA restricted miHA, which could elicit the GVHD in HLA identical HSCT, have no effect on death censored graft survival in kidney transplantation (13). As identified by a genome-wide association study (GWAS), a mismatch in SNP rs17473423 between donor and recipient was associated with acute GVHD development (12). However, the GWAS-identified locus did not predict amino acid alterations, and the precise pathophysiologic mechanism remains to be determined.

In HLA mismatched transplantations, antigen-presenting cells from the recipient can process and present exogenous antigens, including donor-derived HLA and miHA, to $CD4^+$ T helper cells, and initiate graft rejection (14). Similarly, $CD8^+$ T cells of the recipient can recognize donor derived peptides, including a SNP not present in the recipient, as a miHA presented by a matched HLA class I antigen on the donor organ. The effect of miHA at the genomic level has not been assessed in the field of kidney transplantation.

In the current study, we applied GWAS analysis on 300 kidney transplantations, attempting to identify mismatching of genomic missense SNPs between the donor and the recipient, and test the relevance in relation to acute rejection and allograft function.

Materials and Methods

Patients and donors

Patients receiving a renal allograft between 1994 and 2012 at the Leiden University Medical Center (LUMC) were investigated (N=325). A total of 644 DNA samples were investigated (325 patients and 319 donors) comprehending 325 transplant cases, whereby six donors donated two kidneys. Twenty-five transplant cases were excluded because of poor quality of DNA (quality control call rate in the GWAS < 95%).

The case group was defined as having at least one biopsy proven acute rejection (BPAR) episode. Controls were defined as patients having stable graft function without any indication of clinical rejection. Twenty-six transplant cases were excluded since they had an episode of BPAR after switching of maintenance medication or they had clinical indication of rejection with no evidence of BPAR (Figure 1). A total of 232 controls and 42 cases with BPAR were analyzed in the database.

The Modification of Diet in Renal Disease (MDRD) was used to estimate glomerular filtration rate (GFR). In this study, eGFR at 2 years below 40 mL/min/1.73m^2 was used as the cutoff for inferior graft function, since it significantly predicts inferior long term graft survival (Figure S1). A total of 70 patients with inferior graft function and 214 patients with normal graft function were analyzed in the current study.

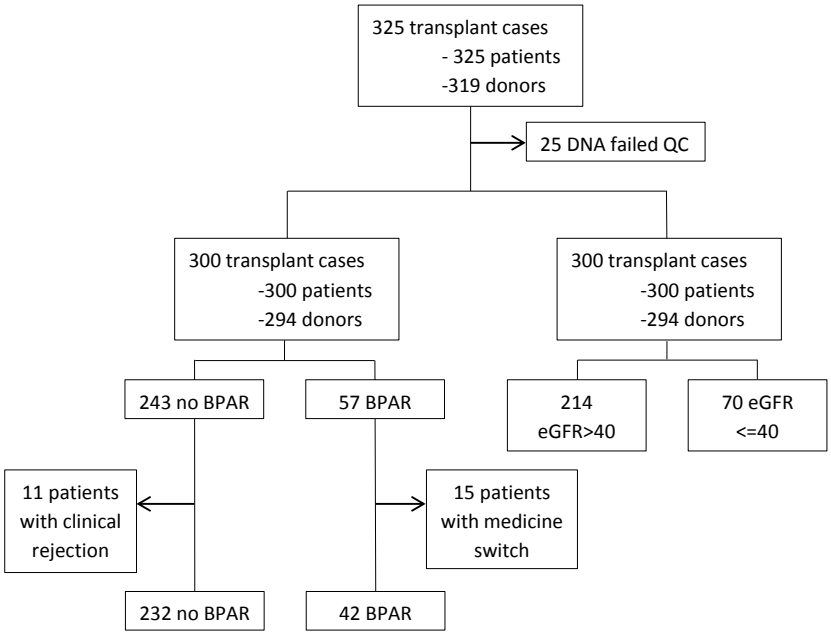


Figure 1: Flow chart of transplant cases included. QC, quality control; BPAR, biopsy proven acute rejection; eGFR, estimated glomerular filtration rate.

Genotyping

Patient and donor DNA was isolated using chemagic DNA Blood2k Kit by chemagic MSM I equipment (PerkinElmer), and the quantity was measured on a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc, Asheville NC). DNA samples were diluted to 50 ng/µl with nuclease-free water. DNA samples were genotyped by transplant SNP array on the Affymetrix platform, which contains 753182 SNPs specially including transplant specific content and function variants module.

Quality control

Genotyping data quality control was performed by Axiom analysis suite based on the 621 individuals (quality control call rate >= 95%). A total of 152,414 SNPs associated with missense SNP or splice site of gene were extracted. SNPs were filtered out using following parameters: SNPs with less than 95% of individuals successfully genotyped; SNPs with a minor allele frequency (MAF) lower than 5%; SNPs significantly (P<0.001) deviating from Hardy-Weinberg equilibrium; marker alleles poorly clustered (Fisher Linear Discriminant <3.6); SNPs without reference SNP identifier (ID); SNPs with missing value in more than 5% of transplant cases. This eventually resulted in 10,800 SNPS for analysis.

Mismatching definition of genomic missense SNP

In the current study, mismatching was considered only when recipient had a homozygous genotype and the donor was either heterozygous or homozygous for the other allele (Table S1). A composite score was calculated, reflecting the total amount of mismatching for the missense SNPs between donor and recipient, termed as mismatch load. The transplant cases were divided into three groups based on the mismatch load, based on tertiles (high, intermediate, low).

Statistical analysis

Association between genomic missense SNP mismatching and BPAR or inferior graft function (eGFR<=40) were tested using logistic regression corrected for the potential clinical risk factors, including donor age, gender, donor type (DCD, DBD, LRD, LURD), patient age, gender, transplantation number (first transplant), immunosuppressive regimens, HLA -A, -B, -DR matching and primary cytomegalovirus infection using R (3.4.0) package. The Bonferroni method was used to correct for multiple comparisons. The association between mismatch load of missense SNP and BPAR or inferior graft function were analyzed by Pearson chi-squared test. BPAR-free rate curves were created using the Kaplan-Meier method.

Table 1. Demographics of study cohort [1]

Variables	no BPAR (N=232)	BPAR (N=42)	P	eGFR<=40 (N=70)	eGFR>40 (N=214)	P
Recipient age (year)[1]	54 (43.25-60)	50 (36.75-62.25)	0.474	56.5 (44-65)	53 (42-59)	0.019*
Recipient gender (% female)	34.1%	23.8%	0.192	35.7%	30.8%	0.448
First transplant (%)	95.70%	92.9%	0.428	92.86%	95.3%	0.537
Donor age (year) [1]	49 (39-59)	51 (38.75-57.75)	0.829	57.5 (49-66.25)	47.5 (37-57)	<0.001*
Donor gender (% female)	50.4%	64.3%	0.098	61.7%	51.0%	0.146
Donor type (%)			0.049*			0.005*
DCD	32.3%	54.8%		50.0%	29.4%	
DBD	23.3%	14.3%		18.6%	24.3%	
LURD	22.4%	16.7%		22.9%	23.4%	
LRD	22.0%	14.3%		8.6%	22.9%	
Cold ischemic time (h) [1]	17 (13.32-20.18) [a]	16.25 (12.3-21.33) [b]	0.844	17 (13.15-21.2)[e]	17 (13.3-20.24)[f]	0.938
DGF (% within deceased donor)	60.5% [c]	72.4% [d]	0.229	79.2%[g]	52.12%[h]	0.001*
HLA-A mismatching (0/1/2)	67/115/50	15/21/6	0.478	20/35/15	65/108/41	0.902
HLA-B mismatching (0/1/2)	42/143/47	9/20/13	0.198	13/37/21	43/130/41	0.247
HLA-DR mismatching (0/1/2)	68/139/25	8/29/5	0.391	16/41/13	63/135/16	0.025*
Primary cytomegalovirus infection	3.90%	9.5%	0.120	10.00%	3.7%	0.042*
Immunotherapy (%)			<0.001*			<0.01*
MMF, CsA	47.8%	85.7%		70.0%	49.1%	
MMF, Tac	16.8%	11.9%		8.6%	16.4%	
MMF, CNI, steroid	35.3%	2.4%		21.4%	34.6%	

HLA, human leukocyte antigen; DGF, delayed graft function; DCD, donor after cardiac death; DBD, donor after brain death; LURD, Living-unrelated donor; LRD, Living-related donor; MMF, mycophenolate mofetil; CsA, cyclosporine A; Tac, Tacrolimus; CNI, calcineurin inhibitor.

[a, b, c, d, e, f, g, h] data missing for 95[a], 10[b], 103[c], 13[d], 19[e], 91[f], 22[g], 99[h] transplant cases.

[1] numbers reflect interquartile ranges.

*P values were calculated using the Mann-Whitney test, Chi-square test or Fisher's exact test.

Results

Patient characteristics and outcomes

A total of 274 Kidney transplant cases passed the quality control for BPAR analysis. Patient- and donor-related variables (age and gender) were not different between controls and BPAR group (Table 1). The donor type and immunosuppressive regime after kidney transplantation did differ (P<0.05). The DCD donor were more frequently in BPAR group and inferior graft function group. The patients received MMF and CsA had a higher frequency in BPAR group and inferior graft function group. DGF rate (only in deceased donors), HLA mismatch, and younger age of the recipient did not predict the episode of acute rejection.

A total of 284 transplant cases were included for inferior graft function analysis. The donor and recipient age were higher in inferior graft function group than superior graft function group. The donor type and immunosuppressive regime significantly differed between the two groups. DGF rate and HLA-DR mismatching predict inferior graft function at two years (Table 1).

Missense SNP mismatching association test for BPAR or eGFR progression

After quality control, 10,800 genomic missense variants were tested for association with BPAR using logistic regression corrected for clinical factors. Of these, 584 showed an association with BPAR, with a P<0.05 (Table S2). After correction for multiple comparisons, none of these variants by themselves were significantly associated with BPAR. Similarly, a total of 596 missense SNPs showed association with low eGFR at P<0.05 (Table S3), whereas none of these SNPs were significantly associated after correction for multiple comparisons. Lack of significant association suggests that the effect of mismatching of single genomic mutations is not strong enough to be detected in this cohort.

Quantitative analysis of genomic missense SNP mismatching

A composite score, reflecting the total amount of mismatching for the missense variants between donor and recipient, was calculated. This score is termed as the mismatch load. As expected, the living related donor (LRD)-recipient combinations had a significantly lower number of genomic mismatching compared to unrelated donor-recipient combinations (Figure 2). The frequency of acute rejection episodes in the LRD group was the same as in the living unrelated donor (LURD) and donor after brain death (DBD) groups (Table 1).

The transplant cases were assigned to one of three groups (tertiles) based on the degree of mismatching with their respective donors. One-third of transplant cases showing the highest degree of mismatching were designated as the ‘more mismatching’ group. Along the same line, the other transplant cases fell in the ‘intermediate mismatching’ or ‘less mismatching’ group (Figure 2). The mismatch load of genomic missense SNPs was not associated with the occurrence of biopsy proven acute rejection and inferior graft function (eGFR<=40) in either all groups together or with the LRD group excluded (Table 2).

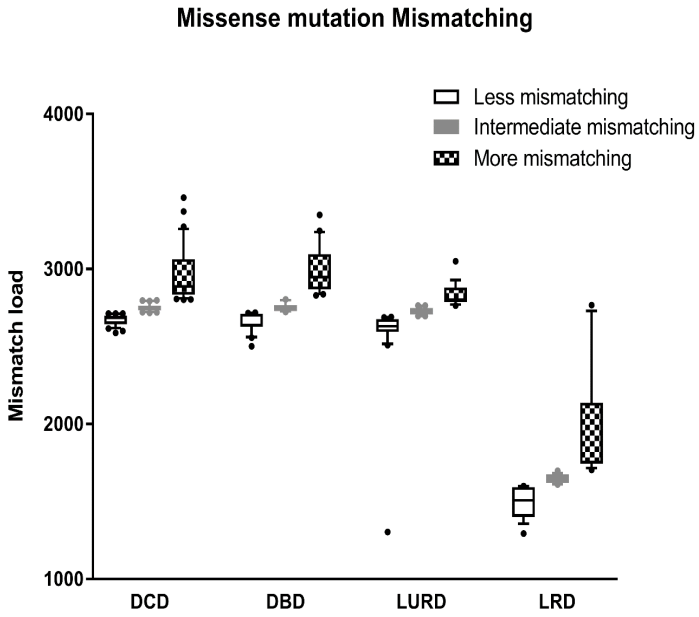


Figure 2: Mismatch load in different donor recipient combinations. Transplant cases were assigned to one of three groups (tertiles) based on the mismatch load: 'more mismatching' group with highest amount of mismatching; 'intermediate mismatching' group with medium amount of mismatching, and 'less mismatching' group with lowest amount of mismatching. Mismatch load of LRD was smaller than in the other donor-recipient combination groups. Box and whisker plots show medians with 10-90 percentile. LRD, living related donors; LURD, living unrelated donors; DBD, donors after brain death; DCD, donors after cardiac death.

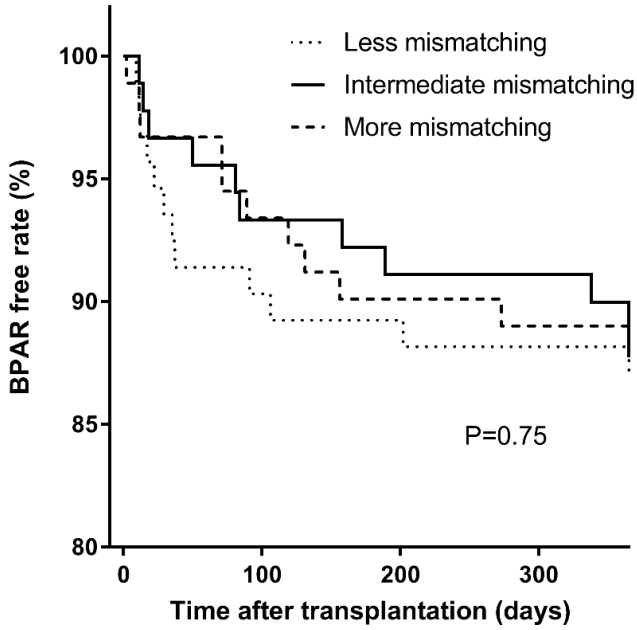
Table 2. Association of genomic mismatch load with acute rejection

Groups	no BPAR (N)	BPAR (N)	P	eGFR<=40(N)	eGFR>40(N)	P
LRD			0.86			0.68
Less mismatching	16	3		3	15	
Intermediate mismatching	17	2		2	17	
More mismatching	18	1		1	17	
DCD,DBD,LURD			0.93			0.67
Less mismatching	61	13		22	54	
Intermediate mismatching	59	12		23	52	
More mismatching	61	11		19	59	
Total cases			0.75			0.57
Less mismatching	77	16		25	69	
Intermediate mismatching	76	14		25	69	
More mismatching	79	12		20	76	

DCD, donor after cardiac death; DBD, donor after brain death; LURD, Living-unrelated donor; LRD, Living-related donor

P values were calculated using Chi-square test or Fisher's Exact Test.

Association of the mismatch load with BPAR-free rate (time to BPAR) at 1 year after transplantation was tested. There was no association between mismatch load and 1 year BPAR-free rate (Figure 3).



Less mismatching	93	85	84	82
Intermediate mismatching	90	85	83	83
More mismatching	91	86	83	82

Figure 3: Association between the mismatching load of missense SNPs and BPAR-free rate. The mismatching load of missense SNPs showed no association to BPAR-free rate.

Association of the analysis of mismatch load under HLA restriction

In fully HLA compatible transplantations, acute rejection may be initiated by miHA differences. In the current study, a series of associations were performed under particular HLA matching conditions. No association was seen between mismatch load and BPAR or inferior graft function in any of the HLA matching subset groups. In full HLA-A-B-DR matching conditions, only two patients had acute rejection, and none of them fell in the 'more mismatching' group. Although the number of transplant cases under HLA matching is limited, the mismatch load did not show a clear association trend with acute allograft rejection or allograft function (Table 3).

Table 3. Association of genomic mismatch load with acute rejection under HLA matching restriction

Groups	no BPAR (N)	BPAR (N)	P	eGFR<=40(N)	eGFR>40(N)	P
HLA-A matching			0.52			0.48
Less mismatching	24	8		8	21	
Intermediate mismatching	20	3		7	18	
More mismatching	23	4		5	26	
HLA-B matching			0.62			0.56
Less mismatching	16	5		7	16	
Intermediate mismatching	19	3		4	17	
More mismatching	7	1		2	10	
HLA-DR matching			0.18			0.29
Less mismatching	23	2		5	20	
Intermediate mismatching	28	6		9	24	
More mismatching	17	0		2	19	
HLA-DQ			0.5			0.18
Less mismatching	37	6		10	36	
Intermediate mismatching	36	7		14	29	
More mismatching	36	3		6	33	
HLA-A+B matching			0.71			1
Less mismatching	11	4		4	12	
Intermediate mismatching	9	2		2	9	
More mismatching	5	0		1	6	
HLA-A+B+DR matching			1			1
Less mismatching	9	1		2	10	
Intermediate mismatching	7	1		1	6	
More mismatching	2	0		1	3	

P values were calculated using Chi-square test or Fisher's Exact Test.

Discussion

Acute allograft rejection remains a complication that negatively affects kidney transplant outcome. Although HLA represents a major contributor in the immune response, non-HLA immunity may also contribute to allograft rejection (10, 15). We investigated the possible clinical relevance of the miHA mismatches, characterized as genomic missense SNPs, in kidney transplantation.

Originally, the miHA were identified using cytotoxic T lymphocytes isolated from the recipient with GVHD after HLA identical bone marrow transplantation (11, 13, 16-18). For allorecognition by $CD8^+$ T cell, the miHA must be presented by those donor HLA antigen, which are shared with the recipient, a phenomenon, which is called HLA restriction (11, 19). The miHA may also be processed and exogenously presented by recipient derived antigen presenting cells to recipient $CD4^+$ T cells, irrespective of the degree of HLA matching between donor and recipient. Therefore, we tested the possible association between mismatched SNPs between donor and recipient with biopsy proven acute rejection under the hypothesis of no HLA restriction.

Genome-wide association studies are a powerful means to identify causal genetic variants associated with phenotype, by analysing millions of SNPs scattered across the genome (14). Several GWAS have been applied to kidney transplantation, leading to identification of novel SNPs in the recipient associated with transplant outcome (20, 21). However, none of the studies investigated the effect of genetic mismatching between the donor and recipient. In HSCT, a large GWAS identifying allele mismatches with acute GVHD were performed in 1,589 unrelated bone marrow transplants matched for HLA-A, -B, -C, -DRB1, and -DQ1 loci (12). Three discrete positive loci, associated with varying grades of GVHD, were identified under HLA restriction, but none of the SNPs predicted amino acid sequence alterations. The successfully detected loci proved that GWAS can capture the risk allele mismatches relevant with clinical outcome. In order to avoid capturing silent SNPs, we pre-selected the SNPs involving amino acid substitution of encoded proteins, which are considered to generate polymorphic epitopes. Therefore, we performed the genotyping of DNA using transplant specific arrays containing 753K SNPs, of which 152K SNPs related with missense mutation. After strictly filtering, a total of 10,800 SNPs were tested for association with outcome, under the assumption of no HLA restriction. Unfortunately, none of the mismatched missense SNPs could predict BPAR or inferior graft function after multiple corrections, which suggest that the effect of genomic mismatching is not strong enough to detect in the relatively low number of transplants studied.

A higher degree of HLA mismatching leads to an inferior graft survival than HLA matched transplants (7). Therefore, we hypothesized that more mismatching of missense SNPs between donor and recipient leads to a higher chance of rejection. The LRD group indeed showed fewer mismatches, but the frequency of acute rejection was not difference than that of other donor types. Even in subgroup analyses, which divided transplant cases into three group based on mismatch load, the degree of mismatching of genomic variants did not predict BPAR incidence, BPAR-free rate or a poorer graft function. Also when HLA restriction was taken into consideration, the incidence of acute rejection or inferior graft function was not higher in the more mismatched group compared to that in the less mismatched group.

The miHA identified in HSCT studies associated with GVHD were mainly targets for $CD8^+$ T cells and recognized in the context of matched HLA class I antigens. Such mismatches did not show any correlation with kidney transplantation outcome (13). In the current study, we found no association between missense SNPs in the genome with BPAR or inferior graft function in kidney transplants. A reasons for our negative finding may be the fact that the additional effect of mismatching of genomic missense SNPs under the condition of HLA mismatching is very low, taking into consideration the efficient immunosuppressive medication given to the patients. The lack of a significant association between allele mismatching and transplant outcome in our study may also be due to the limited number of transplant cases studied. Therefore, our finding needs to be confirmed in an independent, larger-sized cohort.

In conclusion, we found no effect of genomic missense SNP mismatching on biopsy proven acute rejection and inferior graft function in kidney transplantation.

References

1. Pallardó Mateu LM, Sancho Calabuig A, Capdevila Plaza L, Franco Esteve A. Acute rejection and late renal transplant failure: risk factors and prognosis. Nephrology Dialysis Transplantation. 2004;19(suppl_3):iii38-iii42.
2. El-Zoghby ZM, Stegall MD, Lager D, Kremers WK, Amer H, Gloor J, et al. Identifying specific causes of kidney allograft loss. American Journal of Transplantation. 2009;9(3):527-35.
3. Suthanthiran M, Strom TB. Renal transplantation. New England Journal of Medicine. 1994;331(6):365-76.
4. Nankivell BJ, Alexander SI. Rejection of the kidney allograft. New England Journal of Medicine. 2010;363(15):1451-62.
5. McKenna RM, Takemoto SK, Terasaki PI. Anti-hla antibodies after solid organ transplantation. Transplantation. 2000;69(3):319-26.
6. Karahan GE, Claas FH, Heidt S. B Cell immunity in Solid Organ Transplantation. Frontiers in immunology. 2016;7.
7. Claas FH, Roelen DL, Dankers MK, Persijn GG, Doxiadis II. A critical appraisal of HLA matching in today's renal transplantation. Transplantation Reviews. 2004;18(2):96-102.
8. Persijn GG, Cohen B, Lansbergen Q, D'Amaro J, Selwood N, Wing A, et al. Effect of HLA-A and HLA-B matching on survival of grafts and recipients after renal transplantation. The New England journal of medicine. 1982;307(15):905-8.
9. Gratwohl A, Döhler B, Stern M, Opelz G. HY as a minor histocompatibility antigen in kidney transplantation: a retrospective cohort study. The Lancet. 2008;372(9632):49-53.
10. Opelz G. Non-HLA transplantation immunity revealed by lymphocytotoxic antibodies. The Lancet. 2005;365(9470):1570-6.
11. Goulmy E, Schipper R, Pool J, Blokland E, Falkenburg F, Vossen J, et al. Mismatches of minor histocompatibility antigens between HLA-identical donors and recipients and the development of graft-versus-host disease after bone marrow transplantation. New England Journal of Medicine. 1996;334(5):281-5.
12. Sato-Otsubo A, Nannya Y, Kashiwase K, Onizuka M, Azuma F, Akatsuka Y, et al. Genome-wide surveillance of mismatched alleles for graft versus host disease in stem cell transplantation. Blood. 2015:blood-2015-03-630707.
13. Heinold A, Opelz G, Scherer S, Ruhenstroth A, Laux G, Doehler B, et al. Role of minor histocompatibility antigens in renal transplantation. American Journal of Transplantation. 2008;8(1):95-102.
14. Yang JY, Sarwal MM. Transplant genetics and genomics. Nature Reviews Genetics. 2017;18(5):309-26.
15. Terasaki P. Deduction of the fraction of immunologic and non-immunologic failure in cadaver donor transplants. Clinical transplants. 2003:449-52.
16. Nishida T, Akatsuka Y, Morishima Y, Hamajima N, Tsujimura K, Kuzushima K, et al. Clinical relevance of a newly identified HLA-A24-restricted minor histocompatibility antigen epitope derived from BCL2A1, ACC-1, in patients receiving HLA genotypically matched unrelated bone marrow transplant. British journal of haematology. 2004;124(5):629-35.
17. Akatsuka Y, Warren EH, Gooley TA, Brickner AG, Lin MT, Hansen JA, et al. Disparity for a newly identified minor histocompatibility antigen, HA-8, correlates with acute graft-versus-host disease after haematopoietic stem cell transplantation from an HLA-identical sibling. British journal of haematology. 2003;123(4):671-5.
18. Murata M, Warren EH, Riddell SR. A human minor histocompatibility antigen resulting from differential expression due to a gene deletion. Journal of Experimental Medicine. 2003;197(10):1279-89.
19. Shlomchik WD. Graft-versus-host disease. Nature Reviews Immunology. 2007;7(5):340-52.
20. Ghisdal L, Baron C, Lebranchu Y, Viklický O, Konarikova A, Naesens M, et al. Genome-Wide Association Study of Acute Renal Graft Rejection. American Journal of Transplantation. 2017;17(1):201-9.
21. O'brien RP, Phelan PJ, Conroy J, O'kelly P, Green A, Keogan M, et al. A genome-wide association study of recipient genotype and medium-term kidney allograft function. Clinical transplantation. 2013;27(3):379-87.

Supplementary Data

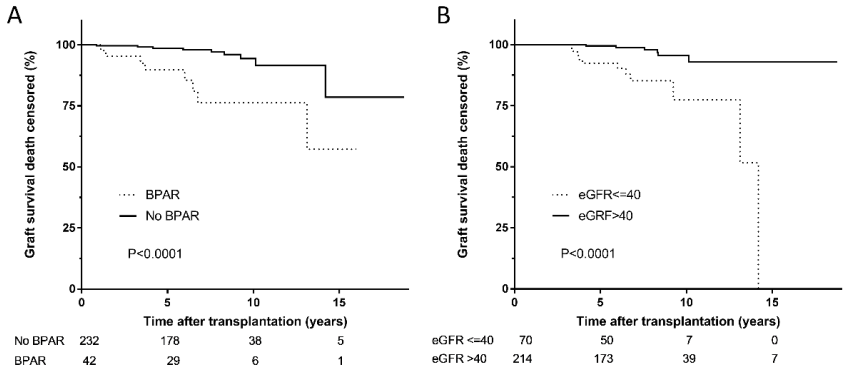


Figure S1: Association of kidney graft survival with the BPAR or eGRF at two year. The BPAR group and progression group with eGFR below 40 mL/min/1.73m^2 significantly predict the long term graft outcome.

Table S1. Mismatching definition of genomic missense SNP

Recipient	Donor	Risk
AA	AA	0
AA	AB	1
AA	BB	1
AB	AA	0
AB	AB	0
AB	BB	0
BB	AA	1
BB	AB	1
BB	BB	0

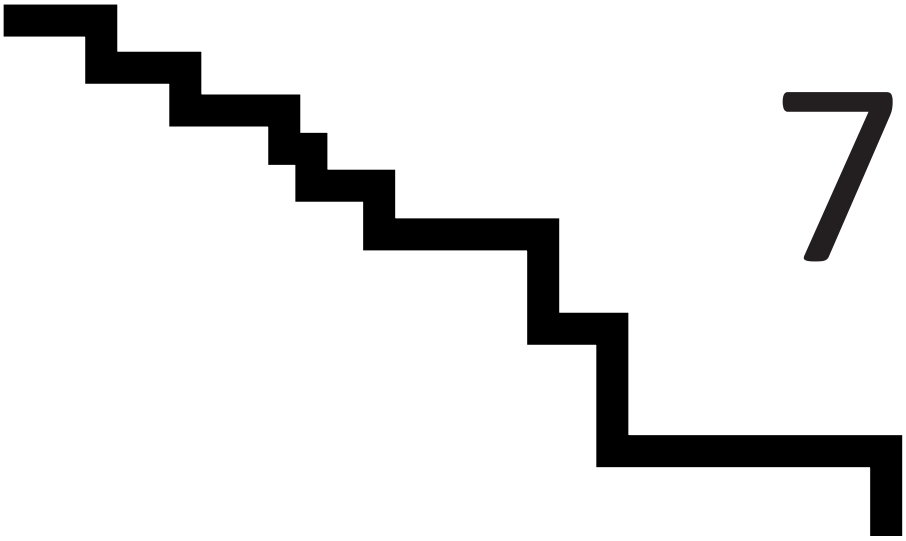
Table S2: Top 20 SNPs significantly associated with biopsy acute rejection without multiple correction.

SNP ID	beta	se	z	P	Odds ratio
rs246105	1.4454	0.4112	3.5154	0.000439	4.243549
rs2234970	-1.6544	0.4734	-3.4945	0.000475	0.191207
rs3877899	1.4996	0.4336	3.4588	0.000543	4.479897
rs2229357	-2.0822	0.6125	-3.3994	0.000675	0.124656
rs2007887	1.5177	0.4476	3.3909	0.000697	4.561721
rs17822931	1.5796	0.4682	3.3738	0.000741	4.853014
rs2305780	1.32	0.4167	3.168	0.001535	3.743421
rs11567842	1.3134	0.4196	3.1303	0.001746	3.718796
rs80131293	1.6839	0.5418	3.1081	0.001883	5.386522
rs4503285	-1.7812	0.5741	-3.1025	0.001919	0.168436
rs2259633	-1.9761	0.6404	-3.0856	0.002031	0.138609
rs2394516	-1.5223	0.4962	-3.0677	0.002157	0.218209
rs7148147	1.4098	0.4648	3.0331	0.002421	4.095136
rs9350797	-1.5592	0.5188	-3.0055	0.002651	0.210304
rs3750266	-1.3735	0.4613	-2.9772	0.002909	0.253219
rs10768450	-1.512	0.5084	-2.974	0.002939	0.220469
rs1191778	-1.5299	0.5177	-2.9551	0.003125	0.216557
rs74643365	1.8219	0.6173	2.9514	0.003163	6.183596
rs628524	-1.7013	0.5777	-2.9449	0.00323	0.182446

Table S3: Top 20 SNPs significantly associated with inferior graft function without multiple correction.

SNP ID	beta	se	z	P	Odds ratio
rs34666677	1.7563	0.4443	3.9527	7.73E-05	5.790971
rs16896629	2.2355	0.6027	3.7092	0.000208	9.351156
rs1233387	-1.472	0.4223	-3.4859	0.000491	0.229466
rs3736228	1.2772	0.3869	3.3009	0.000964	3.586583
rs2071950	-1.317	0.4022	-3.2742	0.00106	0.267938
rs11984293	-1.6732	0.512	-3.2679	0.001083	0.187646
rs11155242	1.2122	0.3776	3.2106	0.001325	3.36087
rs10798035	1.1133	0.3481	3.1985	0.001381	3.044388
rs1131600	-1.5637	0.4906	-3.1876	0.001435	0.20936
rs7426114	-1.3851	0.4363	-3.1745	0.001501	0.250299
rs7614116	-1.2953	0.4084	-3.1714	0.001517	0.273816
rs62399429	-1.6219	0.5134	-3.1593	0.001581	0.197523
rs4745571	1.175	0.3739	3.1429	0.001673	3.238143
rs4680	-1.2072	0.3845	-3.1395	0.001692	0.299033
rs41287373	-1.339	0.4271	-3.135	0.001718	0.262108
rs2229362	1.1277	0.3629	3.1079	0.001884	3.088545
rs2257295	-1.2376	0.4033	-3.0682	0.002153	0.29008
rs45535039	1.0662	0.3491	3.0546	0.002253	2.904322
rs17535963	-1.2623	0.4149	-3.0422	0.002349	0.283002

Genome-wide association studies in kidney transplantation: advantages and constraints



7

Jianxin Yang, Frans H.J. Claas, Michael Eikmans

Department of Immunohematology and Blood Transfusion,
Leiden University Medical Center, Leiden, the Netherlands

Transplant Immunology. 2018 Apr 25.

Abstract
Since the discovery of the human leukocyte antigen (HLA) system, the role of HLA molecules in the field of transplantation has been appreciated: better matching leads to better graft function. Since then, the association of other genetic polymorphisms with clinical outcome has been investigated in many studies. Genome-wide association studies (GWAS) represent a powerful tool to identify causal genetic variants, by simultaneously analyzing millions of single nucleotide polymorphisms scattered across the genome. GWAS in transplantation may indeed be useful to reveal novel markers that may potentially be involved in the mechanism of allograft rejection and graft failure. However, the relevance of GWAS for risk stratification or donor selection for an individual patient is limited as is already reflected by the fact that many parameters, significant in one study, cannot be confirmed in another one.

Introduction
Human leukocyte antigen (HLA) matching has a beneficial effect on kidney graft survival (1, 2). In addition, many other candidate genes beyond HLA loci have been reported to affect kidney transplantation (3, 4). Discrepant results among many of those have been reported, although the association between pharmacogenomics and tacrolimus blood concentrations was frequently observed (5).

Genome-wide association study (GWAS) represent an unbiased approach to identify genetic variants, which are associated with human disease. The approach enables analysis of millions of single nucleotide polymorphisms (SNPs) scattered across the genome. GWAS may also provide a robust genomic platform to characterize genetic risk factors of adverse transplant outcome. Here we discuss that GWAS may be applied to identify novel molecules and pathways involved in acute rejection (AR) and to predict transplant outcomes, but that the technology has not yet been proven to provide a useful guidance for treatment of the individual patient.

Treatment of recipients after transplantation
Despite the application of efficient immunosuppressive drugs, acute rejection episodes still occur in kidney transplant recipients. A rise in serum creatinine may indicate a decreased graft function and a need of further diagnosis by an allograft biopsy. Pulse corticosteroid therapy is the first line of treatment for acute cellular rejection (6, 7). Antibody therapy, such as antithymocyte globulin (ATG) or alemtuzumab, is a more effective approach to normalize kidney function for patients who have more severe forms of acute rejection and/or who do not respond to the pulse steroid treatment (7). Patients with acute antibody mediated rejection may be treated with plasmapheresis, intravenous immune globulin (IVIG) or rituximab (7). Recipients with viral disease after transplantation may benefit from a reduction in dosage of immunosuppression (7).

Irrespective of the type of treatment, all therapies have been relying on clinical monitoring in blood serum and urine, and diagnostic assessment in allograft biopsies, rather than on genetic diversity between individuals.

HLA and transplant outcome

The HLA antigens are the most important histocompatibility antigens involved in alloimmune responses. T cell mediate rejection (TCMR), characterized by the presence of T cells and inflammatory cells in the interstitium and tubular epithelium of the allograft, may be triggered by three distinct mechanisms. Direct allorecognition is driven by the direct interaction between the T cell receptor on recipient T cells and mismatched HLA antigens on donor derived antigen presentation cells (APC). In this process, activated CD4+ T cells produce inflammatory cytokines and CD8+ cytotoxic T cells directly destruct the allograft. At a later time point after transplantation the indirect allorecognition pathway becomes more dominant, whereby donor-derived antigens are processed and presented by recipient APCs to recipient CD4+ T cells (8). Recipient dendritic cells transferred with intact donor HLA can also prime recipient T cells via the semidirect pathway (9). B cells can be activated after recognizing foreign HLA to differentiate to plasma cells and produce donor specific antibodies. These may lead to allograft destruction, which is termed as antibody mediate rejection (ABMR). The presence of antibodies against donor-specific HLA and of C4d deposition in the tissue represent strong evidence for the diagnosis of ABMR (10). It is important to recognize that TCMR may be encountered as a single entity and as a mixed form with features of ABMR (11).

Matching for the HLA-A, HLA-B, and HLA-DR loci has been recognized as great importance for outcome after organ transplantation (12). The beneficial effect of HLA matching were challenged by high graft survival rate in living donors (13). However, the significant effect of HLA matching was still observed under the umbrella of efficient immunosuppressive therapy (2). Therefore, HLA typing and matching remain crucial for graft and patient survival.

Non-HLA genetics of transplant outcome

Terasaki estimated that only 18% of graft loss at 10 years for cadaveric donors can be explained by HLA-related immunologic factors, whereas 38% was caused by non-HLA factors and 43% by non-immunological factors (14). One non-HLA-related risk factor is represented by the human H-Y antigen: a male donor allograft to a female recipient is associated with elevated risk of graft loss after kidney transplantation (15, 16). The MHC class I polypeptide-related sequence A (MICA) represent potential non-HLA antigens that may elicit an antibody production. Transplant recipients with pre-existing anti-MICA antibody are reported to have an inferior one year graft survival (17). A number of studies have shown that the presence of non-HLA antibodies, as identified by protein microarray, is associated with allograft

injury (18-20). In HLA compatible kidney transplantations, mismatching for killer-cell immunoglobulin-like receptors (KIR) and ligands was associated with inferior long term graft survival (21). In a larger independent study, the effect of KIR-ligand mismatching could not be verified (22).

Pharmacogenetics involves the study of genetic variants in drug metabolizing enzymes and transporters. The relationship between SNPs in the drug metabolizing factor CYP3A5 and tacrolimus trough levels in the blood of transplanted patients has been widely described in literature. Hence, dosing adjustments of tacrolimus should be adjusted according to the CYP3A5 genotype, in order to achieve optimal therapeutic concentrations and to reduce tacrolimus toxicity (23, 24). However, pharmacogenetic tests are hardly adopted in transplant centers to optimize the starting dose of immunosuppression. One of the reasons may be the lack of a relevant impact of pharmaco-genotyping test on transplant outcomes (25-28). On the other hand, therapeutic drug monitoring is widely accepted to correct for the effect of pharmacogenetic polymorphisms (29).

Most genetic association studies in kidney transplantation have been focused on SNPs located within or flanking the genes encoding for proteins that play a pivotal role in immune responses, including cytokines, chemokines, toll-like receptors, ficolins, and complement components (3, 30-35). Overviews of genetic variants investigated in relation to transplant outcome, especially occurrence of acute rejection, have been reviewed previously (3-5). Many genetic studies have led to observation of a significant association between candidate SNPs and transplant outcome, but validation of the clinical impact of the same SNPs in follow-up studies often led to inconsistent results. For example, transplant recipients with the complement C3S/S variant (common allele) receiving a kidney allograft with the uncommon variant C3F/F or C3F/S had a beneficial graft outcome, but a larger collaborative study showed that genotypic distribution of C3 alleles does not significantly influence kidney transplantation outcome (34, 35). 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. Currently, no singular candidate SNP has unambiguously shown an association with transplant outcome in both a sufficiently large discovery and validation cohort.

GWAS in transplantation

The candidate SNP approach, as described above, does not provide complete coverage of all possible variants in the genome, and may be limited to genes with a known or postulated involvement in rejection. GWAS enable simultaneous analysis of millions of SNPs spanning the entire genome, which may provide novel insight in the genetic susceptibility of rejection.

Until this moment, GWAS has been performed occasionally in the transplantation field. In 326 Irish kidney transplant recipients, who received a graft from a deceased donor, O'Brien and colleagues reported the association of two genetic variants with five-year graft

function (36). However, in a validation study of 1,638 Caucasians transplant recipients no association of these two particular SNPs could be found with serum creatinine levels and long term graft survival (37). This highlights the importance of validation in genetic association studies and expansion of sample size, for example by international collaboration, to limit false discovery rates.

A large collaborative GWAS of mostly Belgian and French origin, including 778 European kidney transplant recipients, led to identification of two risk loci associated with TCMR, using a DNA pooling approach (38). Two variants were identified (rs10846175 and rs7976329) located in the first intron of protein tyrosine phosphatase receptor type O and one variant (rs10765602) located upstream of coiled-coil domain containing 67, which may play a role in signal transduction in the immune synapse. The authors did not determine the precise mechanism how these SNPs act locally or distantly on genes that are involved in the allo-immune response. Furthermore, the pooled DNA approach may not efficiently reduce the standard deviation of an allele frequency, in case confirmation is not performed by genotyping on individual DNA samples (39). Unfortunately, in our GWAS in 279 kidney transplant recipients (unpublished), a cohort for which we calculated to have sufficient power for validation, we could not confirm the association of these SNPs with biopsy proven acute rejection.

GWAS in African-American kidney transplant recipients led to the identification of two novel CYP3A5 variants (rs10264272 and rs41303343), which were associated with tacrolimus trough levels (23). The number of loss-of-function alleles were related to increased one year eGFR, but not to acute rejection incidence (23). Other GWAS in kidney and in heart transplantation have shown association with occurrence of new-onset diabetes after transplantation (NODAT) and cutaneous squamous cell carcinoma after transplantation (40, 41). GWAS in bone marrow transplantation were mainly focused on acute GvHD and minor HLA antigens, providing evidence that genetic disparity is associated with rejection (42). Unfortunately, minor HLA antigen disparities identified in identical hematopoietic stem-cell transplantation have no effect on death censored graft survival in kidney transplantation (43).

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. A small pilot study showed that the number of amino acid mismatches in trans-membrane proteins was negatively correlated with long term allograft function, independent of HLA matching and donor age (44). Other on-going GWAS in kidney transplantation combined analysis of recipient and donor genomes, such as homozygous loss–of-function variants and nonsynonymous SNP mismatching (45). These efforts may provide novel insight in the mechanism of rejection.

GWAS: limitations and requirements

One of the main limitations to GWAS is the requirement of stringent significance thresholds due to multiple testing, with typically required P-value of less than 5×10^{-8} for single SNPs. Fulfilment of such requirement helps in limiting false positive discoveries, but it also considerably reduces the power to detect associated SNPs. The only way to overcome this limitation is to increase the sample size. However, an intrinsic problem associated with a large multicentre GWAS in transplantation is the fact that donor selection and clinical protocols, including kind and dose of immunosuppression, will differ, which certainly may affect the outcome. Another drawback is that individual genetic variants, implicated by GWAS, have only a small effect on complex traits (46). Riancho pointed out that, even after combining all available GWAS from databases on a particular trait, the polymorphisms identified only explain less than 10% of the susceptibility to the disease (47). In other words, it seems impossible to explain a complex trait with the aid of a few genetic polymorphisms. A third remark concern the fact that the biological function of many variants identified by GWAS, which are mostly located in none-coding regions of the genome, is unknown. Thus, follow-up mechanistic studies would be required to elucidate the role of genetic variants in the process of allograft rejection.

Overall, GWAS represent a powerful approach to identify genetic variants associated with clinical transplant outcome on the population level, and to further expand our knowledge of the mechanism of rejection and graft failure for developing novel treatment strategies. Risk assessment for the individual patient using this technology is difficult. At present, GWAS approaches have not provided a useful guidance in daily clinical practice for personalized treatment of the transplanted patient.

Acknowledgements

J. Yang was awarded financial support from the China Scholarship Council (201306170038).

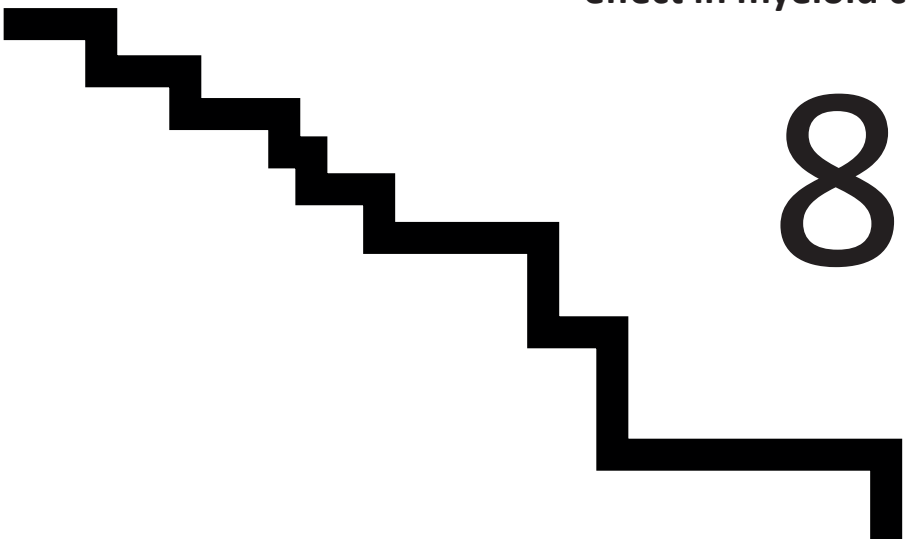
Reference

1. Persijn GG, Cohen B, Lansbergen Q, D'Amaro J, Selwood N, Wing A, et al. Effect of HLA-A and HLA-B matching on survival of grafts and recipients after renal transplantation. New England Journal of Medicine. 1982;307(15):905-8.
2. Claas FH, Roelen DL, Dankers MK, Persijn GG, Doxiadis II. A critical appraisal of HLA matching in today's renal transplantation. Transplantation Reviews. 2004;18(2):96-102.
3. Almoguera B, Shaked A, Keating B. Transplantation genetics: current status and prospects. American Journal of Transplantation. 2014;14(4):764-78.
4. Goldfarb-Rumyantzev AS, Naiman N. Genetic predictors of acute renal transplant rejection. Nephrology Dialysis Transplantation. 2010;25(4):1039-47.
5. Dorr CR, Oetting WS, Jacobson PA, Israni AK. Genetics of Acute Rejection after Kidney Transplantation. Transplant International. 2017.
6. Ganji M, BOROUMAND B. Acute cellular rejection. 2007.
7. Kasiske BL, Zeier MG, Chapman JR, Craig JC, Ekberg H, Garvey CA, et al. KDIGO clinical practice guideline for the care of kidney transplant recipients: a summary. Kidney international. 2010;77(4):299-311.
8. Wood KJ, Goto R. Mechanisms of rejection: current perspectives. Transplantation. 2012;93(1):1-10.
9. Smyth LA, Herrera OB, Golshayan D, Lombardi G, Lechler RI. A novel pathway of antigen presentation by dendritic and endothelial cells: Implications for allorecognition and infectious diseases. Transplantation. 2006;82:S15-S8.
10. Loupy A, Haas M, Solez K, Racusen L, Glotz D, Seron D, et al. The Banff 2015 kidney meeting report: current challenges in rejection classification and prospects for adopting molecular pathology. American Journal of Transplantation. 2017;17(1):28-41.
11. Randhawa P. T-cell-mediated rejection of the kidney in the era of donor-specific antibodies: diagnostic challenges and clinical significance. Current opinion in organ transplantation. 2015;20(3):325-32.
12. Takemoto S, Port FK, Claas FH, Duquesnoy RJ. HLA matching for kidney transplantation. Human immunology. 2004;65(12):1489-505.
13. Terasaki PI, Cecka JM, Gjertson DW, Takemoto S. High survival rates of kidney transplants from spousal and living unrelated donors. New England Journal of Medicine. 1995;333(6):333-6.
14. Terasaki P. Deduction of the fraction of immunologic and non-immunologic failure in cadaver donor transplants. Clinical transplants. 2003:449-52.
15. Gratwohl A, Döhler B, Stern M, Opelz G. HY as a minor histocompatibility antigen in kidney transplantation: a retrospective cohort study. The Lancet. 2008;372(9632):49-53.
16. Kim SJ, Gill JS. HY incompatibility predicts short-term outcomes for kidney transplant recipients. Journal of the American Society of Nephrology. 2009;20(9):2025-33.
17. Zou Y, Stastny P, Süsal C, Döhler B, Opelz G. Antibodies against MICA antigens and kidney-transplant rejection. New England Journal of Medicine. 2007;357(13):1293-300.
18. Sutherland SM, Li L, Sigdel TK, Wadia PP, Miklos DB, Butte AJ, et al. Protein microarrays identify antibodies to protein kinase Cζ that are associated with a greater risk of allograft loss in pediatric renal transplant recipients. Kidney international. 2009;76(12):1277-83.
19. Sigdel TK, Li L, Tran TQ, Khatri P, Naesens M, Sansanwal P, et al. Non-HLA antibodies to immunogenic epitopes predict the evolution of chronic renal allograft injury. Journal of the American Society of Nephrology. 2012;23(4):750-63.
20. Jackson AM, Sigdel TK, Delville M, Hsieh S-C, Dai H, Bagnasco S, et al. Endothelial cell antibodies associated with novel targets and increased rejection. Journal of the American Society of Nephrology. 2015;26(5):1161-71.
21. van Bergen J, Thompson A, Haasnoot G, Roodnat J, de Fijter J, Claas F, et al. KIR-ligand mismatches are associated with reduced long-term graft survival in HLA-compatible kidney transplantation. American Journal of Transplantation. 2011;11(9):1959-64.
22. Tran T, Unterrainer C, Fiedler G, Döhler B, Scherer S, Ruhenstroth A, et al. No Impact of KIR-Ligand Mismatch on Allograft Outcome in HLA-Compatible Kidney Transplantation. American Journal of Transplantation. 2013;13(4):1063-8.
23. Oetting W, Schladt D, Guan W, Miller M, Remmel R, Dorr C, et al. Genomewide association study of tacrolimus concentrations in African American kidney transplant recipients identifies multiple CYP3A5 alleles. American Journal of Transplantation. 2016;16(2):574-82.

24. Rojas L, Neumann I, Herrero MJ, Boso V, Reig J, Poveda JL, et al. Effect of CYP3A5* 3 on kidney transplant recipients treated with tacrolimus: a systematic review and meta-analysis of observational studies. The pharmacogenomics journal. 2015;15(1):38.
25. Glowacki F, Lionet A, Buob D, Labalette M, Allorge D, Provôt F, et al. CYP3A5 and ABCB1 polymorphisms in donor and recipient: impact on Tacrolimus dose requirements and clinical outcome after renal transplantation. Nephrology Dialysis Transplantation. 2011;26(9):3046-50.
26. Hesselink DA, van Schaik RH, van Agteren M, de Fijter JW, Hartmann A, Zeier M, et al. CYP3A5 genotype is not associated with a higher risk of acute rejection in tacrolimus-treated renal transplant recipients. Pharmacogenetics and genomics. 2008;18(4):339-48.
27. Bandur S, Petrasek J, Hribova P, Novotna E, Brabcova I, Viklicky O. Haplotypic structure of ABCB1/MDR1 gene modifies the risk of the acute allograft rejection in renal transplant recipients. Transplantation. 2008;86(9):1206-13.
28. Flahault A, Anglicheau D, Loriot M-A, Thervet E, Pallet N. Clinical impact of the CYP3A5 6986A> G allelic variant on kidney transplantation outcomes. Pharmacogenomics. 2017;18(2):165-73.
29. Van Gelder T, Van Schaik RH, Hesselink DA. Pharmacogenetics and immunosuppressive drugs in solid organ transplantation. Nature Reviews Nephrology. 2014;10(12):725.
30. Alakulppi NS, Kyllönen LE, Jäntti VT, Matinlauri IH, Partanen J, Salmela KT, et al. Cytokine gene polymorphisms and risks of acute rejection and delayed graft function after kidney transplantation. Transplantation. 2004;78(10):1422-8.
31. Kruger B, Krick S, Dhillon N, Lerner SM, Ames S, Bromberg JS, et al. Donor Toll-like receptor 4 contributes to ischemia and reperfusion injury following human kidney transplantation. Proceedings of the National Academy of Sciences. 2009;106(9):3390-5.
32. Grinyo J, Vanrenterghem Y, Nashan B, Vincenti F, Ekberg H, Lindpaintner K, et al. Association of four DNA polymorphisms with acute rejection after kidney transplantation. Transplant International. 2008;21(9):879-91.
33. Eikmans M, de Canck I, van der Pol P, Baan CC, Haasnoot GW, Mallat MJ, et al. The functional polymorphism Ala258Ser in the innate receptor gene ficolin-2 in the donor predicts improved renal transplant outcome. Transplantation. 2012;94(5):478-85.
34. Brown KM, Kondeatis E, Vaughan RW, Kon SP, Farmer CK, Taylor JD, et al. Influence of donor C3 allotype on late renal-transplantation outcome. New England Journal of Medicine. 2006;354(19):2014-23.
35. Varagunam M, Yaqoob MM, Döhler B, Opelz G. C3 polymorphisms and allograft outcome in renal transplantation. New England Journal of Medicine. 2009;360(9):874-80.
36. O'brien RP, Phelan PJ, Conroy J, O'kelly P, Green A, Keogan M, et al. A genome-wide association study of recipient genotype and medium-term kidney allograft function. Clinical transplantation. 2013;27(3):379-87.
37. Pihlstrøm HK, Mjøen G, Mucha S, Haraldsen G, Franke A, Jardine A, et al. Single Nucleotide Polymorphisms and Long-Term Clinical Outcome in Renal Transplant Patients: A Validation Study. American Journal of Transplantation. 2017;17(2):528-33.
38. Ghisdal L, Baron C, Lebranchu Y, Viklický O, Konarikova A, Naesens M, et al. Genome-Wide Association Study of Acute Renal Graft Rejection. American Journal of Transplantation. 2017;17(1):201-9.
39. Sham P, Bader JS, Craig I, O'Donovan M, Owen M. DNA pooling: a tool for large-scale association studies. Nature Reviews Genetics. 2002;3(11):862.
40. Sanders ML, Karnes JH, Denny JC, Roden DM, Ikizler TA, Birdwell KA. Clinical and genetic factors associated with cutaneous squamous cell carcinoma in kidney and heart transplant recipients. Transplantation direct. 2015;1(4).
41. McCaughan JA, McKnight AJ, Maxwell AP. Genetics of new-onset diabetes after transplantation. Journal of the American Society of Nephrology. 2013:ASN. 2013040383.
42. Sato-Otsubo A, Nannya Y, Kashiwase K, Onizuka M, Azuma F, Akatsuka Y, et al. Genome-wide surveillance of mismatched alleles for graft versus host disease in stem cell transplantation. Blood. 2015:blood-2015-03-630707.
43. Heinold A, Opelz G, Scherer S, Ruhenstroth A, Laux G, Doehler B, et al. Role of minor histocompatibility antigens in renal transplantation. American Journal of Transplantation. 2008;8(1):95-102.
44. Mesnard L, Muthukumar T, Burbach M, Li C, Shang H, Dadhania D, et al. Exome sequencing and prediction of long-term kidney allograft function. PLoS computational biology. 2016;12(9):e1005088.
45. Reindl-Schwaighofer R, Kainz A, Cole B, van Setten J, Jelencsics K, Keating B, et al., editors. Alloimmunity

through non-HLA epitopes in kidney transplantation. American journal of transplantation; 2016: Wiley-blackwell 111 river st, hoboken 07030-5774, nj USA.
46. Goldstein DB. Common genetic variation and human traits. New England Journal of Medicine. 2009;360(17):1696.
47. Riancho JA. Genome-wide association studies (GWAS) in complex diseases: advantages and limitations. Reumatología Clínica (English Edition). 2012;8(2):56-7.

Calcium-binding proteins S100A8 and S100A9: investigation of their immune regulatory effect in myeloid cells



8

Jianxin Yang, Jacqueline Anholts, Ulrike Kolbe, Janine A. Stegehuis-Kamp, Frans H. J. Claas and Michael Eikmans*

Department of Immunohematology and Blood Transfusion, Leiden University Medical Center

International Journal of Molecular Sciences. 2018, 19(7), 1833.

Abstract

High expression levels of the calcium-binding proteins S100A8 and S100A9 in myeloid cells in kidney transplant rejections are associated with a favorable outcome. Here we investigated the myeloid cell subset expressing these molecules, and their function in inflammatory reactions. Different monocyte subsets were sorted from buffy coats of healthy donors and investigated for S100A8 and S100A9 expression. To characterize S100A9high and S100A9low subsets within the CD14+ classical monocyte subset, intracellular S100A9 staining was combined with flow cytometry (FACS) and qPCR profiling. Furthermore, S100A8 and S100A9 were overexpressed by transfection in primary monocyte-derived macrophages and the THP-1 macrophage cell line to investigate the functional relevance. Expression of S100A8 and S100A9 was primarily found in classical monocytes and to a much lower extent in intermediate and non-classical monocytes. All S100A9+ cells expressed human leukocyte antigen - antigen D related (HLA-DR) on their surface. A small population (<3%) of CD14+CD11b+CD33+HLA-DR− cells, characterized as myeloid derived suppressor cells (MDSCs), also expressed S100A9 to high extent. Overexpression of S100A8 and S00A9 in macrophages led to enhanced extracellular reactive oxygen species (ROS) production, as well as elevated mRNA expression of anti-inflammatory *IL-10*. The results suggest that the calcium-binding proteins S100A8 and S100A9 in myeloid cells have an immune regulatory effect.

Introduction

The S100 calcium-binding proteins A8 and A9 (S100A8 and S100A9), also known as migration inhibitory factor-related proteins 8 (MRP8) and 14 (MRP14), are abundantly expressed in myeloid cells, such as circulating monocytes and neutrophils. Their level of expression can be used as a biomarker of inflammation in bacterial infections and autoimmune diseases (1-5). At the protein level S100A8 and S100A9 can form a heterodimeric complex, which is called calprotectin and which has antimicrobial effects (6-8). Extracellular S100A8 and S100A9 proteins bind to Toll-like receptor 4 and the receptor for advanced glycation end products (RAGE) to trigger NF-κB activation and production of proinflammatory cytokines and chemokines (9, 10).

In contrast, several reports have provided evidence that S100A8 and S100A9 can exert anti-inflammatory effects: this was shown in lipopolysaccharides (LPS)-induced endotoxemia and autoimmune myocarditis (11, 12). Intracellular S100A9 can regulate adaptive immunity by inducing accumulation of myeloid-derived suppressor cells (MDSCs) in tumor-bearing individuals (13-15). MDSCs are able to suppress T cell responses. Chen and colleagues showed that S100A9 inhibits the differentiation of dendritic cells and macrophages and induces accumulation of MDSCs through elevated reactive oxygen species (ROS) production (14). Sinha and colleagues reported that S100A8/A9 binds to glycoprotein receptors on MDSCs and promotes their migration and accumulation (15). S100A9 was proposed as a novel marker of human monocytic MDSCs (16).

The role of myeloid cells in allograft rejection has been increasingly appreciated (17-19). We found in human kidney transplants that acute rejection episodes with high tissue expression of *S100A8* and *S100A9* have a more favorable long-term outcome than rejections with low expression (20, 21), suggesting that the S100 proteins exert beneficial immune effects. Double immunofluorescence on tissue biopsies showed that S100A9 largely co-localized with CD68 and HLA-DR, but that only a minority of S100A9+ cells expressed the macrophage type 2 marker CD163. This suggests that S100A9+ cells infiltrating the graft represent a distinct macrophage subset that potentially can interact with T cells through their surface HLA class II molecules. Furthermore, both in peripheral blood mononuclear cells (PBMC) and biopsies, we observed correlations of *S100A9* expression with the expression of *CD11b* and *CD33* (21). The combination of high CD11b and CD33 and low HLA-DR is used by flow cytometry to distinguish MDSCs (22). MDSCs have been observed to accumulate in kidney transplant recipients, and they were able to induce expansion of regulatory T cells in vitro (23, 24). Furthermore, patients with high numbers of MDSCs in their blood at time of acute transplant rejection had a favorable graft outcome (24).

Based on previous findings we hypothesize that S100A9+ myeloid cells have distinct immune regulatory properties. In the current study, we phenotypically characterized monocytes that differentially expressed S100A8 and S100A9, and identified a functional role of these calcium-binding proteins in macrophages.

Materials and Methods

Reagents

The following reagents were used for the study: phosphate-buffered saline (PBS, 10×) pH 7.4 (ThermoFisher-Ambion, Cat. No. AM9625, Vilnius, Lithuania), distilled water (DNase/RNase free, Gibco, Cat. No. 10977-035), paraformaldehyde (PFA) (EM Grade, Purified, Electron Microscopy Sciences, Hatfield, PA, USA), Saponin (Sigma-Aldrich, Saint Louis, MO, USA), recombinant ribonuclease inhibitor (ThermoFisher-Invitrogen, Cat. No. 10777-019, Carlsbad, CA, USA), bovine serum albumin (BSA) fraction V-molecular biology grade (Gemini bio-products, Cat. No. 700-106P, Burgess Hill, UK), PE mouse anti-human S100A9 Clone 1H9 (BD bioscience-Pharmingen, Cat. No. 565793, San Diego, CA, USA), rabbit-anti-human S100A9 (ab92507, Abcam, Cambridge, UK), goat-anti-rabbit IgG-AF488 (A11008, Invitrogen, Bleiswijk, The Netherlands), RPMI1640 (Gibco, Grand island, NY, USA), fetal calf serum (FCS), penicillin, streptomycin, L-glutamine (Gibco, Invitrogen, Carlsbad, CA, USA), diphenyleneiodonium (DPI; Sigma-Aldrich, D2926, Saint Louis, MO, USA), GM-CSF (Thermo-Fisher, PHC2013-1MG, Carlsbad, CA, USA), mouse anti-human antibody against CD14, CD16, and HLA-DR (all BD Bioscience).

Monocyte purification

Written informed consent was obtained from donors for use of buffy coats for scientific purposes. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Hypaque density gradient centrifugation from buffy coats of anonymous healthy donors (Sanquin blood bank, Leiden, the Netherlands). CD14+ monocytes were isolated from PBMC by positive selection using human CD14 microbeads (Miltenyi biotec GmbH, Bergisch Gladbach, Germany) according to the manufacturer's instructions.

Monocyte subsets FACS sorting

Purified PBMC were stained with antibodies against CD14 and CD16 in 100 μL of FACS buffer. After washing twice, three monocyte subsets were sorted using FACSAria flow cytometer according to the CD14 and CD16 expression level. Sorted subsets were pelleted and lysed for RNA isolation.

Measurement of S100A9 in monocytes using the cytospin method

CD14 microbeads-enriched monocytes (positive selection) were washed with PBS and resuspended at a concentration of 0.5×10^6 cells/mL. Three drops of cell suspension were transferred onto a glass slide using a cytospin 4 cytocentrifuge (Thermo Scientific) at room temperature. Cells were fixed using cold acetone for 10 min and washed three times. Cells were incubated with a 1000-times-diluted rabbit-anti-human S100A9 antibody for 1 h at room temperature. After washing, slides were incubated with 1:200 diluted goat-anti-rabbit

IgG-AF488 for 30 min. Images were acquired on a fluorescence microscope (Axioskop 40, Carl Zeiss, Jena, Germany) using digital imaging software (ZEN 2.3 lite, Carl Zeiss, Jena, Germany).

Intracellular staining and FACS sorting

Intracellular staining procedures, followed by RNA extraction and qPCR, were mainly based on a previous study (25). In brief, one million CD14+ monocytes were fixed and permeabilized with 4% PFA and 1% saponin in 250 μL of PBS, supplemented with 1–5% RNase inhibitor for 30 min at 4oC. Cells were pelleted and washed once using 1 mL of wash buffer (0.2% BSA, 0.1% saponin, 2.5% RNase inhibitor in PBS) at 4oC. Cells were incubated for 30 min at 4oC with 5 μL of mouse anti-human S100A9 antibody in 50 μL of staining buffer (1% BSA, 0.1% saponin, 5% RNase inhibitor in PBS). Cells were washed twice with 1 mL of wash buffer and resuspended in sorting buffer (0.5% BSA, 5% RNase inhibitor in PBS). Cells were sorted into two subsets based on S100A9 protein expression using a FACSAria flow cytometer (BD Biosciences). Intracellular staining used for flow cytometric analysis were performed using the BD Cytofix/Cytoperm kit (BD Biosciences, Cat.No. 554714)

RNA isolation and cDNA synthesis

After flow cytometric sorting, the cell suspension was centrifuged at 3000g for 5 min at 4oC and the supernatant was removed. Total RNA from intracellular stained cells was isolated using the RecoverAll Total Nucleic Acid Isolation Kit for FFPE (Ambion, AM1975, Carlsbad, CA, USA), by incubating with 100 μL of protease digestion buffer for 3 h at 50 °C. The other steps in the RNA isolation procedure were carried out according to the manufacturer's instructions. When working with unstained, intact cells, total RNA was extracted using the NucleoSpin miRNA kit (Macherey-Nagel, Düren, Germany). Complementary DNA synthesis was performed as described previously (21, 26).

Real-time quantitative PCR

Primer sequences for quantitative PCR are provided in Table S1. To prevent amplification of genomic DNA, reverse and forward primers were designed to target separate exons, spanning at least one intron with a size of 800 bp or more. All primer sets were tested on control cDNA, and PCR efficiencies were between 90% and 110%. Relative gene expression levels were normalized to the geometric mean of the reference genes β-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Overexpression of S100A8/A9 and measurement of ROS and cytokines

To allow differentiation into macrophages, CD14+ monocytes were cultured in the presence of 5 ng/mL GM-CSF for 5 days. Monocyte-derived macrophages were seeded at 1.5×10^6 per well in 6-well plates. After 5 days, transfection mixture containing 20 μL of Lipofectamine

2000 (Thermo Fisher) and 7.5 μg plasmid was added dropwise to the wells. After 5–6 h the medium was replaced by fresh complete roswell park memorial institute (RPMI) medium and incubated for another 24 h. The extracellular ROS production by the cells was measured as described previously (21).

The THP-1 macrophage cell line was transfected with similar amounts of plasmid as described above, and incubated for 24 h. Cells were washed and resuspended for incubation for another 24 h in complete medium, either containing no stimulants or containing 100 ng/mL LPS. The cells were then pelleted and lysed for RNA isolation.

Statistical analyses

Statistical analyses were performed using SPSS statistics 23 and GraphPad Prism 7.02. All experiments were performed at least three times. The statistical difference between the two groups was calculated by *t*-test, and the difference between the two groups was calculated by one-way ANOVA with Tukey's multiple comparison tests.

Results

S100A9 is mostly expressed in CD14-positive (classical) monocytes

S100A9 expression levels were assessed in three monocyte subsets, designated as classical (CD14+ CD16−), intermediate (CD14+ CD16+), and non-classical (CD14− CD16+) monocytes (Figure 1A). Messenger RNA analysis of *CD14* and *CD16* in the three sorted populations verified the sorting strategy (Figure 1B). Expression of S100A9 was most abundant in the classical monocytes (Figure 1B), which encompassed at least 75% of the total monocyte population (Figure 1A). Protein expression of S100A9 by flow cytometry was seen in all three monocyte subsets, and it was higher than that seen in lymphocytes (Figure 1C,D). The median fluorescence intensity (MFI) of S100A9 in classical and intermediate monocytes was approximately twice as high as that of non-classical monocytes (Figure 1D). The results show that S100A9 is mostly expressed in CD14-positive monocytes.

S100A9 expression varies within the CD14+ monocyte population

Next, we tested whether there is variation in S100A9 expression within the CD14+ monocyte population. For this, we subjected CD14+ enriched cells to cytospin analysis of S100A9 protein. The fluorescence intensity varied greatly between cells (Figure 1E). Similarly, the fluorescence-activated cell sorting (FACS) plot showed a wide range of S100A9 expression within the CD14+ classical monocytes (Figure 1C).

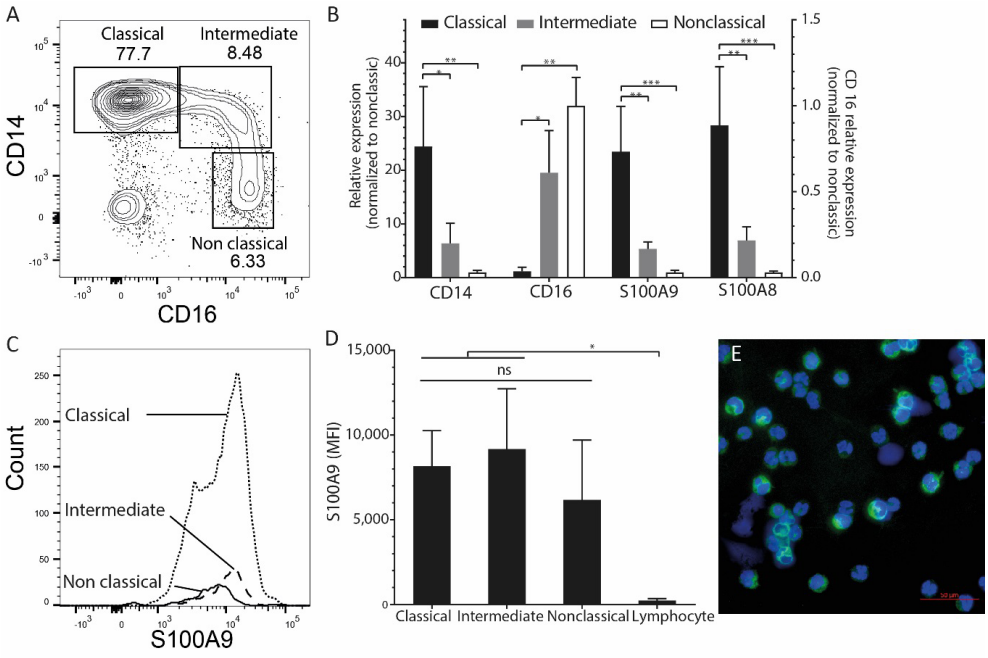


Figure 1. S100A9 expression is highest in CD14+ classical monocytes. (A) Classical, intermediate, and nonclassical monocytes subsets were sorted based on CD14 and CD16 expression using FACS; (B) The relative expression of S100A9 in the classical subset was 20-fold higher than that in the non-classical subset; (C) The representative FACS histogram plot showed that S100A9 expression in the three monocyte subsets overlapped with each other; (D) The median fluorescence intensity (MFI) of S100A9 in the classical subset was approximately twice as high than that in the non-classical subset; (E) The cytospin results showed that the fluorescence intensity varied greatly between individual cells within the CD14+ monocyte population; scale bar: 50 µm. The differences were tested by one-way ANOVA with Tukey's multiple comparison tests. Data are expressed as means ± SD of at least three biological replicates. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Both HLA-D-positive monocytes and myeloid derived suppressor cells express S100A9

To investigate whether S100A9-positive monocytes express HLA-DR on their surface and are potentially able to interact with CD4+ T cells, we analyzed S100A9 and HLA-DR expression by FACS on PBMC from healthy donors. HLA-DR and S100A9 were co-expressed in CD14+ monocytes (Figure 2A). HLA-DR-low monocytes showed slightly higher expression of S100A9 than HLA-DR-high monocytes in healthy donors (Figure 2B,C). The results show that all S100A9-positive monocytes express HLA-DR on their surface.

We further observed that MDSCs, which are characterized as CD14+CD11b+CD33+HLA-DR− and constitute only a small percentage of the total monocyte population (Figure 2D), showed an even higher S100A9 expression than the CD14+HLA-DR+ subset, although this difference was not significant (Figure 2E).

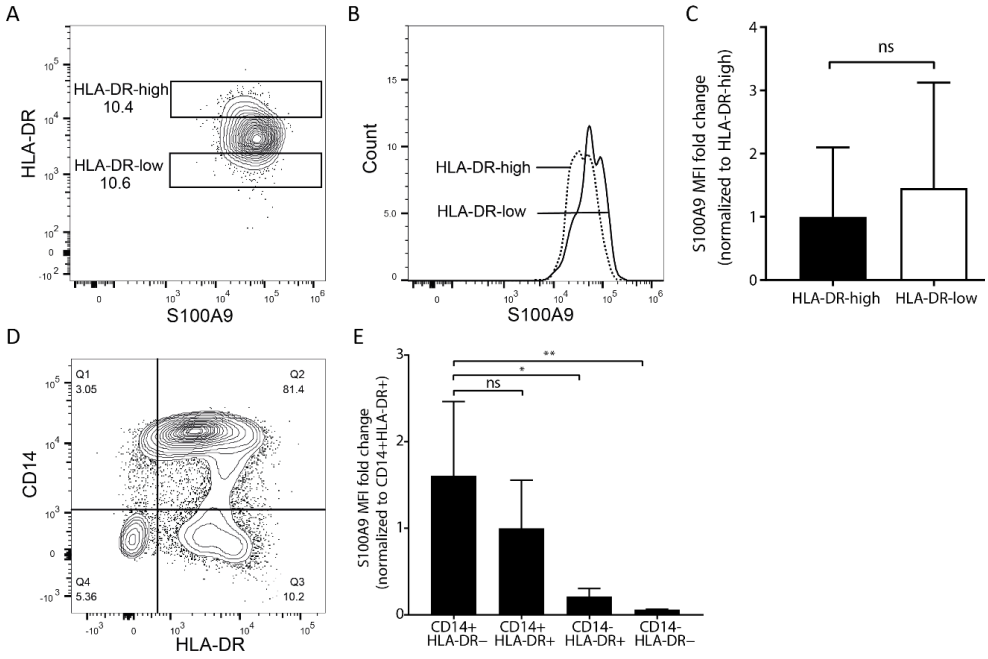


Figure 2. S100A9-positive monocytes express HLA-DR. Peripheral blood mononuclear cells (PBMC) were stained for CD14, HLA-DR, and S100A9, and were gated on CD14+ cell populations. (A) Representative FACS plot showing that CD14+ monocytes express both HLA-DR and S100A9. Gates were set to the upper and lower 10% of HLA-DR expression for the CD14+ monocytes, to further study subsets; (B,C) S100A9 expression was not significant between HLA-DR-high and HLA-DR-low subsets, although, S100A9 was slightly higher in the latter subset. Significant differences were calculated by *t-test*; (D) Representative FACS plot showing expression of CD14 and HLA-DR in gated CD33+ myeloid cells; (E) Monocytic MDSC (CD14+HLA-DR−) subsets showed slightly higher expression of S100A9 than CD14+HLA-DR+ subsets, but this was not significant. The differences were tested by one-way ANOVA with Tukey's multiple comparison tests. All data are expressed as means ± SD of four different experiments. * $p < 0.05$, ** $p < 0.01$.

Phenotypic characterization of cytokine expression profiles in S100A9high and S100A9low monocytes

To test whether S100A9high and S100A9low monocytes differ phenotypically in their cytokine expression profile, we combined intracellular S100A9 FACS staining with mRNA analysis of sorted cell populations. Since fixation and intracellular FACS staining disturbs the RNA quality inside the cells, the assay needed to be optimized first. Processed (fixed and intracellular stained) cells showed significantly reduced qPCR signals (as indicated by higher Cq values) compared to live cells (Figure 3A). Application of a buffer containing 5% RNase inhibitor greatly improved RNA quality, as detected by a significant decrease in the Cq value by qPCR (Figure 3A).

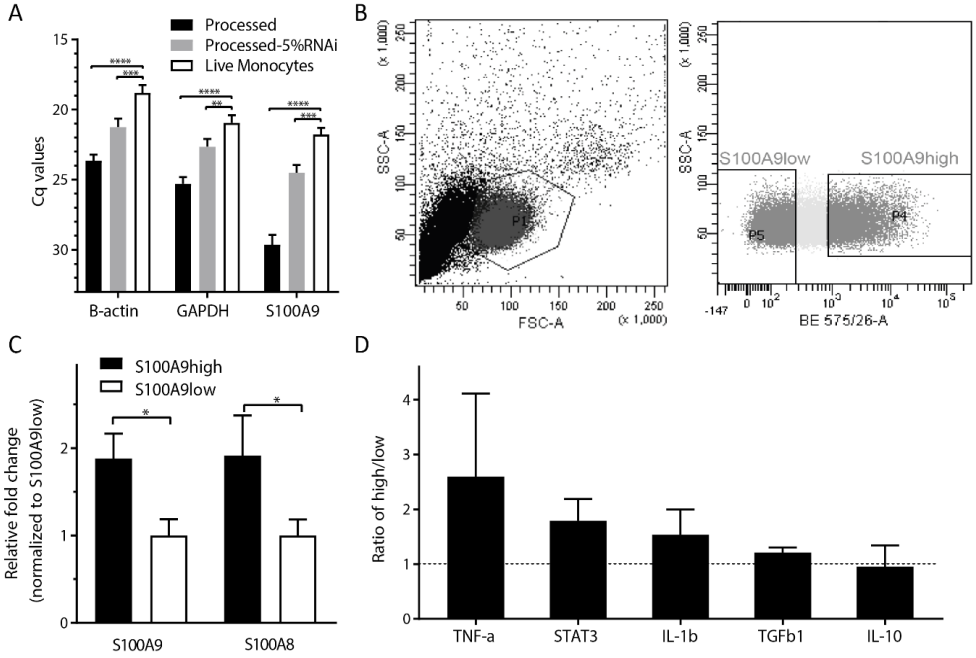


Figure 3. S100A9high and S100A9low subsets of classical monocytes do not differ in their expression of immune-related cytokines. (A) The addition of increased RNAse inhibitor concentration (5%) to lysis buffer minimizes the extent of RNA degradation (delta Cq < 34). Differences were tested by one-way ANOVA with Tukey's multiple comparison tests; (B) Monocytes were sorted into two subsets based on the S100A9 protein level: S100A9high and S100A9low; (C) Relative gene expression of S100A9 from FACS sorted cell populations based on S100A9 protein levels. The S100A9high subset expressed significantly higher S100A9 mRNA levels than the S100A9low subset. The difference was tested by *t-test*; (D) The relative mRNA expression ratio of pro- and anti-inflammatory cytokines was not significantly different between S100A9 high and S100A9 low subsets. The dashed line indicates relative mRNA expression ratio between S100A9 high and S100A9 low subsets is one. The differences were tested using one sample *t-test*. All data are expressed as means ± SD of three different experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

The monocytes were then sorted into two subsets based on their S100A9 protein level: S100A9high and S100A9low (Figure 3B). S100A9high subsets expressed significantly higher S100A9 mRNA levels than S100A9low subsets (Figure 3C). Several cytokines and growth factors were measured at the RNA level in the S100A9high and S100A9low subsets, but we did not observe significant differences between the subsets in expression levels of acute phase proteins *TNFα, IL-1β*, and of anti-inflammatory cytokines *TGFβ1* and *IL-10* (Figure 3D).

Sorting of viable cells: application of SmartFlare

To sort viable monocytes expressing S100A9 for further functional analysis, SmartFlare RNA detection probes were applied. The probes, attached to inert nanogold particles, are taken up by the cells and emit a fluorescent signal upon binding to their target S100A9 mRNA transcripts within the cells. Isolated CD14+ monocytes were incubated with different amounts of SmartFlare probes for 16 h. The scrambled control probe, which does not recognize any mRNA sequence within the cells, generated a strong fluorescence signal indicating a high level of background staining with all tested concentrations (Figure S1A). Monocytes incubated with the SmartFlare probes were sorted into three groups based on the Cy5 fluorescence intensity (Figure S1B), and the level of S100A9 expression was then validated in each of these three subsets by qPCR and intracellular staining using anti-S100A9 monoclonal antibody. The Cy5high monocyte subset showed no difference of *S100A9* mRNA expression in comparison to the Cy5intermediate and Cy5low subsets (Figure S1C). The intracellular S100A9 protein expression was comparable among the three sorted cell subsets (Figure S1D). Therefore, functional analysis of the monocyte subsets that differentially express S100A9 could not be performed because of unreliable detection by the SmartFlare probes.

Overexpression of S100A8 and S100A9 in monocyte-derived macrophages leads to increased ROS production and elevated IL-10 mRNA expression

To investigate the functional consequence of intracellular S100A9, and its counterpart S100A8, a plasmid containing the full S100A8 and S100A9 sequences was transfected into monocyte-derived macrophages. Transfection led to a 20–40-fold overexpression of S100A8 and S100A9 mRNA (Figure 4A). Overexpression of S100A8 and S100A9 in the monocyte-derived macrophages led to a threefold increase of ROS production (Figure 4B,C). The inhibition of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity by diphenylene iodonium (DPI) normalized the elevated ROS levels in the transfected macrophages, showing that S100A8/S100A9-induced ROS production was exerted through NADPH activation. To examine the effect of ROS-producing macrophages on T cells, macrophages were co-cultured with allogeneic T cells. Unfortunately, transfection of the empty control plasmid already led to decreased induction of T cell stimulation compared to

untransfected macrophages, suggesting that the transfection procedure interferes with the macrophages' T cell stimulating capacity.

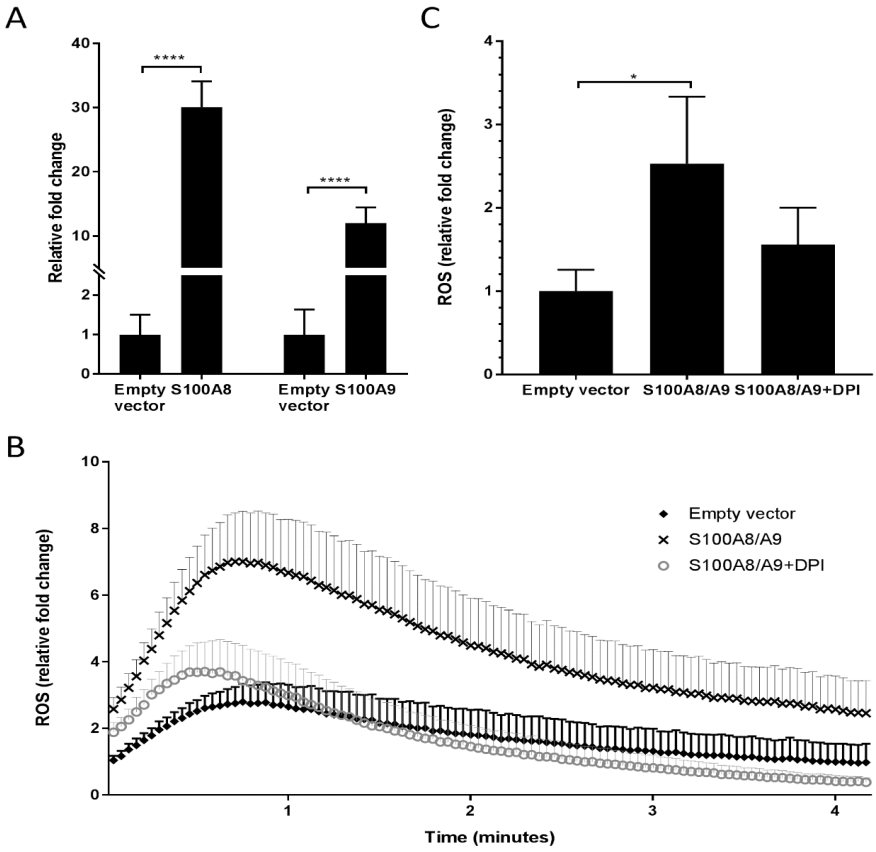


Figure 4. Overexpression of S100A8/A9 in monocyte-derived macrophage increases reactive oxygen species (ROS) production. (A) The mRNA level in monocyte-derived macrophages after transfection was significantly higher than that in the empty plasmid control. The differences were tested by *t-tests*; (B) After phorbol 12-myristate 13-acetate (PMA) activation, the transfected cells were placed immediately in a luminometer to measure their ROS production real-time every 2.5 s. Data are expressed as means ± SEM of at least four different experiments; (C) The peak of ROS production, assessed at the tenth timepoint (25 s) was significantly higher in the S100A8/A9-overexpressed macrophages compared to cells transfected with the empty plasmid. Inhibition of NADPH oxidase activity by diphenylene iodonium (DPI) blocked the ROS production. The differences were tested by one-way ANOVA with Tukey's multiple comparison tests. All data are expressed as means ± SD of at four different experiments. * $p < 0.05$, **** $p < 0.0001$.

We next overexpressed the S100 calcium-binding proteins in THP-1 macrophages to study effects on cytokine synthesis by these cells. To first evaluate the transfection efficiency of the cells, a green fluorescent protein (GFP)-containing plasmid was transfected for 24 h. The high proportion of GFP-positive cells indicates a high transfection efficiency (Figure 5C). We did observe that transfection with an empty vector already caused slight changes compared to non-transfected cells both with respect to GFP signals (Figure 5A,B) and cell viability, which led us to compare the differences between the empty vector and S100A8/A9 transfection conditions. Overexpression of S100A8 and S100A9 in macrophages, both in unstimulated cells and in cells that were stimulated by LPS after the transfection, led to a consistent increase (2-fold to 64-fold) in IL-10 mRNA expression compared to the empty vector transfection condition (Figure 5D). At the same time, expression of pro-inflammatory cytokines TNFα and IL-1β did not change. Excreted cytokines in the supernatants were assessed by Luminex assays, but only TNFα could be detected, which showed no difference between the S100A8/A9 overexpressed cells and the control conditions.

Discussion

Calcium-binding proteins S100A8 and S100A9 are involved in various inflammatory disorders. Relatively high levels of S100A8 and S100A9 in kidney biopsies have a beneficial effect on long-term graft outcome, independent of the extent of myeloid cell infiltration. In this study, we phenotypically characterized the monocyte subsets that differentially express S100A8 and S100A9. We further showed that overexpression of S100A8 and S100A9 in macrophages leads to enhanced ROS production and IL-10 expression.

Monocytes have been documented to consist of three main cell populations based on the expression level of CD14 and CD16: classical, intermediate, and nonclassical (27-29). At the mRNA level, S100A8 and S100A9 were abundantly expressed in the classical monocyte subset (28, 30, 31). At the protein level, the three subsets were more similar in their expression, with a small trend for the non-classical monocytes to express the lowest levels. In line with an earlier study where proteomic and transcriptomic methods were used, CD16-negative monocytes had higher mRNA and protein levels of S100A9 than the CD16-positive subsets (31). We demonstrated a heterogeneous expression of S100A9 within the CD14+ monocyte population, which was the basis for the hypothesis that monocytes with high levels of S100A9 exert distinct immune regulatory effects.

As the expression of S100A9 in the CD14+HLA-DR-low subset was even slightly higher than that in the HLA-DR-high subset, it is clear that HLA-DR is not an appropriate cell surface marker for sorting monocytes that differently express S100A9 protein. Consistent with our earlier findings in transplant tissue (21), S100A9 and HLA-DR are co-expressed in peripheral CD14+ monocytes. Furthermore, the monocytic MDSC subset (CD14+CD11b+CD33+HLA-

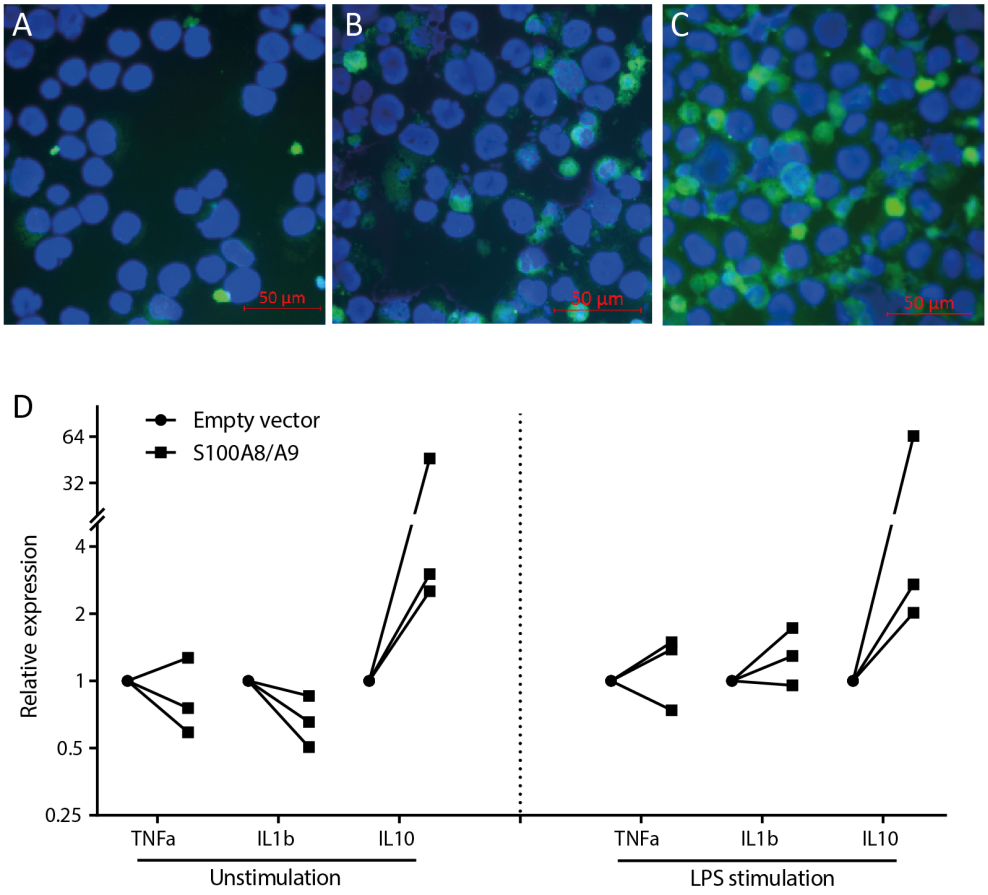


Figure 5. Overexpression of S100A8/A9 in macrophages leads to increased IL-10 expression. (A–C) Validation of transfection efficiency of the THP-1 macrophage cell line. The following cytospins were inspected by immunofluorescence for green light emission: (A) non-transfected cells; (B) cells transfected with an empty plasmid; (C) and those with a GFP-containing plasmid. Scale bar: 50 µm. The results show high transfection efficiency in the cells; (D) Overexpression of S100A8 and S100A9 leads to a consistent increase of IL-10 expression, but not of TNFα and IL-1β. This was observed in both unstimulated cells (left panel) and LPS–stimulated cells (right panel). Data are shown as boxplots (median, upper value, lower value) representing three different experiments.

DR−), which represented a tiny fraction (<3%) of the total myeloid population in our healthy donor material, showed an even higher S100A9 expression than the CD14+HLA-DR+ subset, although this difference was not significant. It does support the earlier findings of S100A9

as both a marker and an inducer of MDSCs. On the basis of previous publications (23), it is expected that in inflammatory situations such as post-transplant conditions, the fraction of MDSCs, and thereby S100A8 and S100A9, rises considerably. The current findings further show that not a single subset, but multiple ones, express the S100 molecules to a relatively high extent.

We investigated S100A9high and S100A9low monocytes, based on intracellular FACS staining, for cytokine expression. Recently, a method for the isolation of high quality RNA from cells following fixation, intracellular staining, and FACS sorting by the use of buffers containing RNase inhibitors has been described (25). However, when following that protocol we observed that the process of intracellular staining still led to reduced RNA quality compared to live, unfixed monocytes. A possible explanation for this discrepancy might be the fact that monocytes contain more ribonuclease A family member 2 (RNASE2) than the human embryonic stem cells, as found by microarray analysis (32), in which the RNA quality was improved by increasing the RNase inhibitor concentration (25). Nevertheless, when normalized to reference gene signals to correct for input and quality differences, we could verify significantly increased *S100A9* mRNA expression in the FACS S100A9high subset compared to the S100A9low subset, which gave confidence with respect to the reliability of the results for the other transcripts studied.

SmartFlare probes have been suggested as suitable tools to detect mRNA in single living cells. Several studies showed successful detection of mRNA using SmartFlare or NanoFlare probes (33-36). In contrast to these publications, in our study the mRNA levels reflected by SmartFlare fluorescence intensity could not be validated by qPCR and flow cytometry. The *S100A9* expression levels were as high as the reference gene (*GAPDH*) and were certainly above the SmartFlare detection limit (Cq < 34). Consistent with our findings, Maria and colleagues demonstrated that SmartFlare fluorescence intensity does not correlate with the level of target transcript, but depends on the efficiency of probe accumulation (37). We found that the SmartFlare fluorescence intensity positively correlated with the forward scatter value, which reflects cell size. Levy and colleagues showed by electron microscopy that the nanoparticle probes remain in intracellular compartments and do not reflect the level of mRNA transcripts in the cytoplasm (38). Mirkin and colleagues, who developed the NanoFlare probes, confirmed that spherical nucleic acid (SNA) localize in late endosomes. They also proposed that the fluorescence signal detected may be due to disassembly of SNA and degradation by the DNaseII (39). This may explain why we observed high background levels in the scrambled probes.

To investigate the functional effect of S100A8 and S100A9, we overexpressed both molecules in primary monocyte-derived macrophages. We did observe that this led to significantly increased ROS production, which was similar to what was found in our previous study using the established, secondary THP-1 macrophage cell line (21). Through this mechanism, the S100 calcium-binding proteins may exert anti-inflammatory effects,

and thereby be beneficial in reducing tissue damage after transplantation. In contrast to THP-1 cells, which lack crucial co-stimulation molecules, monocyte-derived macrophages are able to stimulate (allogeneic) T cells in a mixed lymphocyte culture. When present in the immune synapse during interaction of macrophages with T cells, the extracellular ROS may negatively affect T cell activation and proliferation (40). We observed that the transfer of a large plasmid (~7000 base pairs) into the cells negatively affects cell viability and T cell stimulating potential, which unfortunately held us back from performing mixed lymphocyte cultures between transfected macrophages and T cells. Interestingly, in relation to the link between S100A8/A9 and ROS, the MDSC population in our study was found to express the highest levels of S100A8 and S100A9. MDSCs have been shown to exert T cell inhibiting effects in individuals with a tumor and in those with inflammatory conditions, such as those seen after transplantation (13, 14, 23, 41). S100A9 can be involved, through STAT3 and ROS, in inducing accumulation of MDSCs (13, 14). We did observe that transfection with the empty vector caused some changes compared to non-transfected cells with respect to GFP signals and cell viability, as discussed above. Indeed, it has been described before that green fluorescence production may result from the mere presence of a plasmid and from the use of transfection reagents (42). These observations led us to compare the differences between the empty vector and S100A8/A9 transfection conditions. We found that S100A8/A9-transfected macrophages demonstrate higher expression of the anti-inflammatory cytokine *IL-10*, but not of pro-inflammatory cytokines *TNFα* and *IL-1β*. Despite the consistent *IL-10* mRNA increase in all experiments, a statistically significant difference was not obtained between cells transfected with S100A8/A9 and those transfected with empty vectors, probably because of the variation in the range of increase between experiments. Despite our efforts using protein screening in the supernatant at 24 h after transfection, IL-10 protein could not be detected. Hence, it cannot be stated with certainty that IL-10 is a significant mediator in the induction of immune regulation. Especially in the light of the presence of HLA-DR on S100A9-positive cells, both ROS and IL-10 may represent parts of a pathway through which the S100 molecules trigger the induction of regulatory T cells and inhibition of effector T cells, thereby limiting or preventing a detrimental immune response to the graft.

In summary, the results suggest that calcium-binding proteins S100A8 and S100A9 in myeloid cells have an immune regulatory effect, which may explain the previously observed beneficial effect of the local S100A8 and S100A9 expression on kidney graft survival (20, 21).

Acknowledgments

J.Yang. was awarded financial support from the China Scholarship Council (201306170038).

References

1. Kane D, Roth J, Frosch M, Vogl T, Bresnihan B, FitzGerald O. Increased perivascular synovial membrane expression of myeloid-related proteins in psoriatic arthritis. Arthritis & Rheumatology. 2003;48(6):1676-85.
2. Odink K, Cerletti N, Brüggen J, Clerc RG, Tarcsay L, Zwadlo G, et al. Two calcium-binding proteins in infiltrate macrophages of rheumatoid arthritis. Nature. 1987;330(6143):80-2.
3. Ryckman C, Vandal K, Rouleau P, Talbot M, Tessier PA. Proinflammatory activities of S100: proteins S100A8, S100A9, and S100A8/A9 induce neutrophil chemotaxis and adhesion. The Journal of Immunology. 2003;170(6):3233-42.
4. Pechkovsky D, Zalutskaya O, Ivanov G, Misuno N. Calprotectin (MRP8/14 protein complex) release during mycobacterial infection in vitro and in vivo. FEMS Immunology & Medical Microbiology. 2000;29(1):27-33.
5. Frosch M, Strey A, Vogl T, Wulffraat NM, Kuis W, Sunderkötter C, et al. Myeloid-related proteins 8 and 14 are specifically secreted during interaction of phagocytes and activated endothelium and are useful markers for monitoring disease activity in pauciarticular-onset juvenile rheumatoid arthritis. Arthritis & Rheumatology. 2000;43(3):628-37.
6. Leukert N, Vogl T, Strupat K, Reichelt R, Sorg C, Roth J. Calcium-dependent tetramer formation of S100A8 and S100A9 is essential for biological activity. Journal of molecular biology. 2006;359(4):961-72.
7. Murthy A, Lehrer R, Harwig S, Miyasaki K. In vitro candidastatic properties of the human neutrophil calprotectin complex. The Journal of Immunology. 1993;151(11):6291-301.
8. Sohnle PG, Hunter MJ, Hahn B, Chazin WJ. Zinc-reversible antimicrobial activity of recombinant calprotectin (migration inhibitory factor—related proteins 8 and 14). The Journal of infectious diseases. 2000;182(4):1272-5.
9. Vogl T, Tenbrock K, Ludwig S, Leukert N, Ehrhardt C, Van Zoelen MA, et al. Mrp8 and Mrp14 are endogenous activators of Toll-like receptor 4, promoting lethal, endotoxin-induced shock. Nature medicine. 2007;13(9):1042-9.
10. Boyd JH, Kan B, Roberts H, Wang Y, Walley KR. S100A8 and S100A9 mediate endotoxin-induced cardiomyocyte dysfunction via the receptor for advanced glycation end products. Circulation research. 2008;102(10):1239-46.
11. Otsuka K, Terasaki F, Ikemoto M, Fujita S, Tsukada B, Katashima T, et al. Suppression of inflammation in rat autoimmune myocarditis by S100A8/A9 through modulation of the proinflammatory cytokine network. European journal of heart failure. 2009;11(3):229-37.
12. Ikemoto M, Murayama H, Itoh H, Totani M, Fujita M. Intrinsic function of S100A8/A9 complex as an anti-inflammatory protein in liver injury induced by lipopolysaccharide in rats. Clinica Chimica Acta. 2007;376(1):197-204.
13. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. Nature reviews immunology. 2009;9(3):162-74.
14. Cheng P, Corzo CA, Luetteke N, Yu B, Nagaraj S, Bui MM, et al. Inhibition of dendritic cell differentiation and accumulation of myeloid-derived suppressor cells in cancer is regulated by S100A9 protein. Journal of Experimental Medicine. 2008;205(10):2235-49.
15. Sinha P, Okoro C, Foell D, Freeze HH, Ostrand-Rosenberg S, Srikrishna G. Proinflammatory S100 proteins regulate the accumulation of myeloid-derived suppressor cells. The Journal of Immunology. 2008;181(7):4666-75.
16. Zhao F, Hoechst B, Duffy A, Gamrekelashvili J, Fioravanti S, Manns MP, et al. S100A9 a new marker for monocytic human myeloid-derived suppressor cells. Immunology. 2012;136(2):176-83.
17. Zuidwijk K, de Fijter JW, Mallat MJ, Eikmans M, van Groningen MC, Goemaere NN, et al. Increased influx of myeloid dendritic cells during acute rejection is associated with interstitial fibrosis and tubular atrophy and predicts poor outcome. Kidney international. 2012;81(1):64-75.
18. Toki D, Zhang W, Hor K, Liuwantara D, Alexander S, Yi Z, et al. The role of macrophages in the development of human renal allograft fibrosis in the first year after transplantation. American Journal of Transplantation. 2014;14(9):2126-36.
19. Bergler T, Jung B, Bourier F, Kühne L, Banas MC, Rümmele P, et al. Infiltration of macrophages correlates with severity of allograft rejection and outcome in human kidney transplantation. PloS one. 2016;11(6):e0156900.

20. Eikmans M, Roos-van Groningen MC, Sijpkens YW, Ehrchen J, Roth J, Baelde HJ, et al. Expression of surfactant protein-C, S100A8, S100A9, and B cell markers in renal allografts: investigation of the prognostic value. Journal of the American Society of Nephrology. 2005;16(12):3771-86.
21. Rekers NV, Bajema IM, Mallat MJ, Petersen B, Anholts JD, Swings GM, et al. Beneficial Immune Effects of Myeloid-Related Proteins in Kidney Transplant Rejection. American journal of transplantation. 2016;16(5):1441-55.
22. Bronte V, Brandau S, Chen SH, Colombo MP, Frey AB, Greten TF, et al. Recommendations for myeloid-derived suppressor cell nomenclature and characterization standards. Nat Commun. 2016;7:12150.
23. Luan Y, Mosheir E, Menon M, Wilson D, Woytovich C, Ochando J, et al. Monocytic Myeloid-Derived Suppressor Cells Accumulate in Renal Transplant Patients and Mediate CD4+ Foxp3+ Treg Expansion. American journal of transplantation. 2013;13(12):3123-31.
24. Meng F, Chen S, Guo X, Chen Z, Huang X, Lai Y, et al. Clinical significance of myeloid-derived suppressor cells in human renal transplantation with acute T cell-mediated rejection. Inflammation. 2014;37(5):1799-805.
25. Hrvatin S, Deng F, O'Donnell CW, Gifford DK, Melton DA. MARIS: method for analyzing RNA following intracellular sorting. PloS one. 2014;9(3):e89459.
26. Yang J, Kemps-Mols B, Spruyt-Gerritse M, Anholts J, Claas F, Eikmans M. The source of SYBR green master mix determines outcome of nucleic acid amplification reactions. BMC Res Notes. 2016;9(1):292.
27. Boyette LB, Macedo C, Hadi K, Elinoff BD, Walters JT, Ramaswami B, et al. Phenotype, function, and differentiation potential of human monocyte subsets. PloS one. 2017;12(4):e0176460.
28. Wong KL, Tai JJ-Y, Wong W-C, Han H, Sem X, Yeap W-H, et al. Gene expression profiling reveals the defining features of the classical, intermediate, and nonclassical human monocyte subsets. Blood. 2011;118(5):e16-e31.
29. Ziegler-Heitbrock L, Ancuta P, Crowe S, Dalod M, Grau V, Hart DN, et al. Nomenclature of monocytes and dendritic cells in blood. Blood. 2010;116(16):e74-e80.
30. Ancuta P, Liu K-Y, Misra V, Wacleche VS, Gosselin A, Zhou X, et al. Transcriptional profiling reveals developmental relationship and distinct biological functions of CD16+ and CD16-monocyte subsets. BMC genomics. 2009;10(1):403.
31. Zhao C, Zhang H, Wong W-C, Sem X, Han H, Ong S-M, et al. Identification of novel functional differences in monocyte subsets using proteomic and transcriptomic methods. Journal of proteome research. 2009;8(8):4028-38.
32. Su AI, Wiltshire T, Batalov S, Lapp H, Ching KA, Block D, et al. A gene atlas of the mouse and human protein-encoding transcriptomes. Proceedings of the National Academy of Sciences of the United States of America. 2004;101(16):6062-7.
33. Prigodich AE, Seferos DS, Massich MD, Giljohann DA, Lane BC, Mirkin CA. Nano-flares for mRNA regulation and detection. ACS nano. 2009;3(8):2147-52.
34. Li B, Menzel U, Loebel C, Schmal H, Alini M, Stoddart MJ. Monitoring live human mesenchymal stromal cell differentiation and subsequent selection using fluorescent RNA-based probes. Scientific reports. 2016;6:26014.
35. Halo TL, McMahon KM, Angeloni NL, Xu Y, Wang W, Chinen AB, et al. NanoFlares for the detection, isolation, and culture of live tumor cells from human blood. Proceedings of the National Academy of Sciences. 2014;111(48):17104-9.
36. Seferos DS, Giljohann DA, Hill HD, Prigodich AE, Mirkin CA. Nano-flares: probes for transfection and mRNA detection in living cells. Journal of the American Chemical Society. 2007;129(50):15477-9.
37. Czarnek M, Bereta J. SmartFlares fail to reflect their target transcripts levels. Scientific reports. 2017;7(1):11682.
38. Levy R, Held M, Mason D, Comenge J, Carolan G. The spherical nucleic acids mRNA detection paradox. ScienceOpen Research. 2015.
39. Wu XA, Choi CHJ, Zhang C, Hao L, Mirkin CA. Intracellular fate of spherical nucleic acid nanoparticle conjugates. Journal of the American Chemical Society. 2014;136(21):7726-33.
40. Belikov AV, Schraven B, Simeoni L. T cells and reactive oxygen species. Journal of biomedical science. 2015;22(1):85.
41. Kumar V, Patel S, Tcyganov E, Gabrilovich DI. The nature of myeloid-derived suppressor cells in the tumor microenvironment. Trends in immunology. 2016;37(3):208-20.
42. Guo B, Pearce A, Traulsen K, Rintala A, Lee H. Fluorescence produced by transfection reagents can be confused with green fluorescent proteins in mammalian cells. Biotechniques. 2001;31(2):314-6, 8, 20-1.

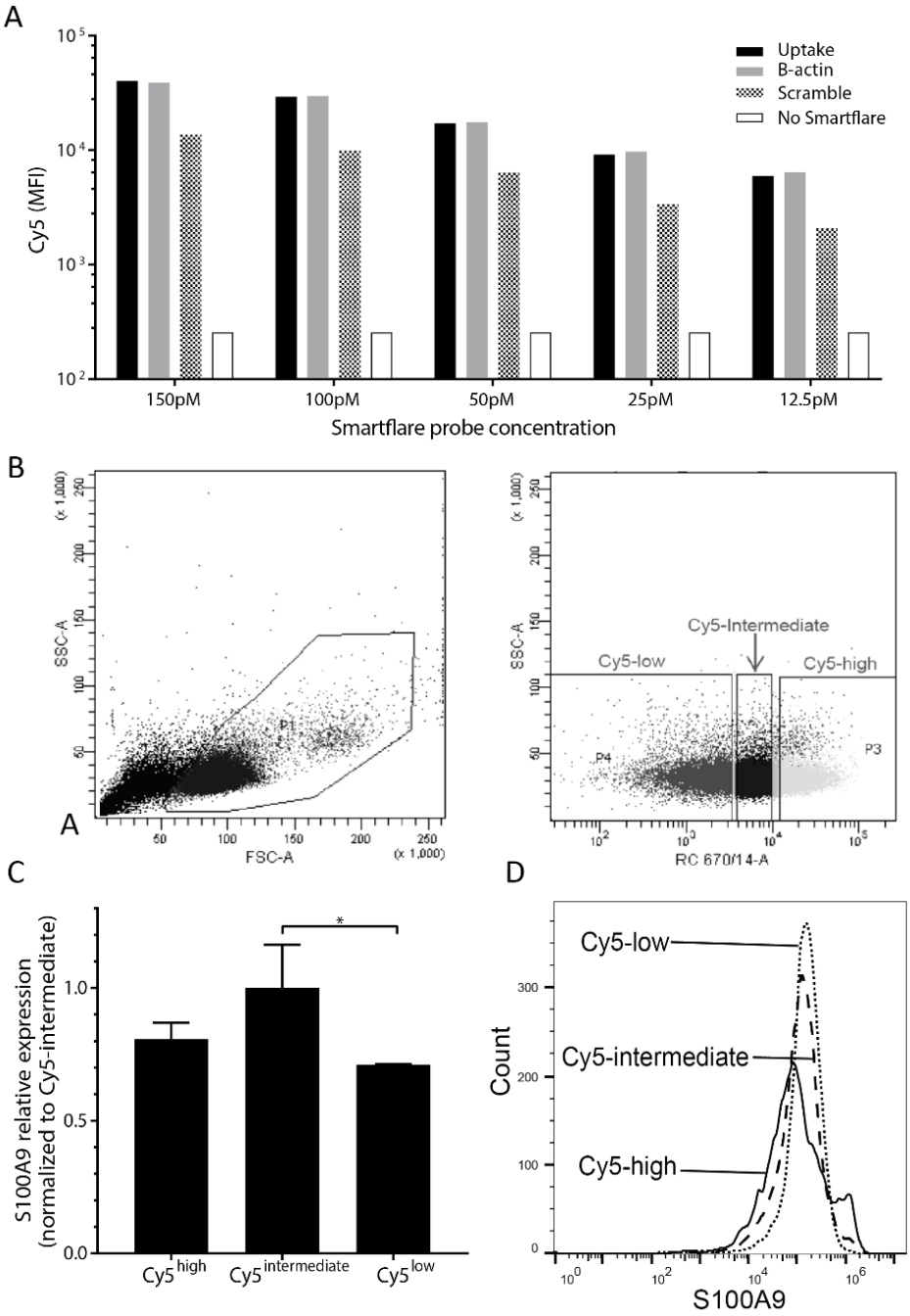
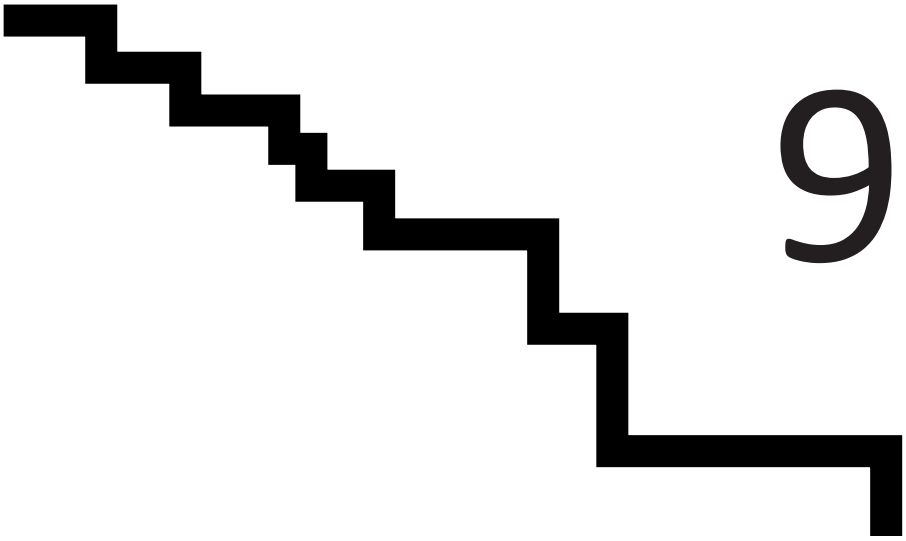

A
Cy5 (MFI)
Uptake
B-actin
Scramble
No Smartflare
150pM
100pM
50pM
25pM
12.5pM
Smartflare probe concentration
B
SSC-A
FSC-A
P1
Cy5-Intermediate
Cy5-low
Cy5-high
P4
P3
RC 670/14-A
C
S100A9 relative expression
(normalized to Cy5-intermediate)
Cy5 high
Cy5 intermediate
Cy5 low
D
Cy5-low
Cy5-intermediate
Cy5-high
Count
S100A9

Figure S1. SmartFlare failed to report the mRNA level in live cells. (A) Monocytes were incubated for 16 hours with different amounts of SmartFlare probe. The scrambled control probe produced strong signals, indicating high background levels. (B) Monocytes were sorted into three subsets based on the Cy5 fluorescence intensity: Cy5-high, Cy5-intermediate, and Cy5-low. (C,D) The mRNA (C) and protein (D) level of S100A9 in three FACS-sorted subsets were not consistent with the SmartFlare signals. Flags show means with SD. Differences were tested by one-way ANOVA with Tukey's multiple comparison tests. Data in panel C are shown as means ± SD of three biological replicates. *P<0.05.

Table S1. Primer sequences used for qPCR

Genes	Forward primer (5'-3')	Reverse primer (5'-3')
B-actin	ACCACACCTTCTACAATGAG	TAGCACAGCCTGGATAGC
GAPDH	ACCCACTCCTCCACCTTTGAC	TCCACCACCCTGTTGCTGTAG
IL-10	GCGCTGTCATCGATTTCTTCC	GTAGATGCCTTTCTCTTGGAGCTTA
IL-1b	TGGCTTATTACAGTGGCAATG	GTGGTGGTCGGAGATTCG
S100A8	GGGAATTTCCATGCCGTCT	CCTTTTTCCTGATATACTGAGGACACT
S100A9	CAGCTGGAACGCAACATAGA	TCAGCTGCTTGTCTGCATTT
STST3	CACGCCTTCTACAGACTG	CATCCTGGAGATTCTCTACC
TGF-b1	CCCAGCATCTGCAAAGCTC	GTCAATGTACAGCTGCCGCA
TNF-a	CCCCAGGGACCTCTCTCTAATC	TACAACATGGGCTACAGGCTTG

Summary and general discussion



9

Summary and general discussion

Kidney transplantation is the preferred treatment of patients with end stage renal disease, as it provides longer patient survival and better quality of life compared to dialysis (1). The allograft survival has been considerably improved over the past decades due to the advancement of surgical procedures, tissue typing, and immunosuppressive medication, especially the introduction of cyclosporine. Potential complications after kidney transplantation, such as delayed graft function and acute rejection (AR), remain risk factors for long-term graft outcome (2-6). Prediction of DGF, response to steroid resistant rejection and long-term graft outcome remain difficult when using merely clinical parameters. Numerous studies have reported on the predictive value of molecular markers for AR and worse graft outcome(7-13). However, the heterogeneity of AR and the variation among transplant centers leads to controversial results and preclude a more general clinical application. In the first part of this thesis, we aimed to investigate the molecular markers of steroid resistance and long-term graft survival on the basis of acute rejection biopsies. In the second part, we focused on genetic variants associated with acute rejection in kidney transplantation. In the final part we described the possible immune regulatory effect of S100 calcium binding proteins.

Selection of SYBR green master mix

Quantitative polymerase chain reaction (qPCR) is a sensitive and specific technique based on non-specific SYBR green chemistry to measure gene expression levels. The extraction and preservation protocols for obtaining high quality and quantity of RNA were optimized in order to increase the sensitivity of mRNA expression assessment (7). The PCR devices and master mixes also affect the accuracy and reliability of the gene expression assays. In chapter 2 we compared different commercial SYBR green PCR mixes using two different PCR machines with respect to the specificity and sensitivity of the qPCR assay.

Three commercial SYBR green PCR mixes: ABI, Bio Rad, and Roche, were tested for 79 immune-related transcripts targeted by specific primer pairs. We found that most primer sets (N=66, 94.3%) could generate a single sharp melting peak with all tested PCR mixes by strictly following the prescribed PCR protocol. However, 13 primer pairs (18.6%) produced suboptimal melting peak using Roche mixes. The use of ABI mixes often led to lower Cq values for cDNA and lower background levels for negative DNA samples compared to the other mixes. The PCR devices had a smaller influence on the results than the source of SYBR green mixes. Based on the data obtained in these studies, we decided to measure all molecular transcripts in biopsy samples using the ABI mix on Viia7 PCR equipment.

Lack of association with DGF at the time of transplantation

Delayed graft function, defined as requirement of dialysis within the first week after transplantation, is a risk factor for acute rejection (4, 5, 14). Ischemia reperfusion injury

(IRI) after transplantation resulting in acute tubular injury is considered as a main cause of DGF (15). Clinical parameters, such as donor creatinine level, prolonged ischemia time, and older recipient age, correlate with DGF (16-18). Accurate prediction of DGF using molecular profiles may allow the early intervention and prevention of further allograft injury. We tested the expression levels of Toll-like receptors (TLRs), complement and apoptosis related genes in pre-implementation biopsies and investigate the relationship with DGF in chapter 3.

In deceased donors none of the markers investigated in pre-implementation biopsies was predictive for the DGF, which is in contrast with a previous study showing that the BAX:BCL2 ratio was elevated in DGF group (19). We found that expression of C2 and C3, and BAX:BCL2 ratio were higher in deceased donors compared to living donors, indicative for a role of the complement and apoptosis pathways in ischemia reperfusion injury. This observation confirmed previous studies showing that complement components are significantly higher in deceased donors compared to living donors (20), and that apoptotic cell death is initiated as reflected by an increased BAX and decreased BCL2 during normothermic ischemia injury (21). Therefore, inhibition of complement and apoptosis pathway may act as therapeutic target to protect from the effects of IRI.

The complement regulators analyzed were not significantly different in their expression between living and deceased donors. TLR2, TLR4 or MyD88 deficient mice are protected from IR injury, and TLR4 expression was significantly higher in pre-implantation biopsies from deceased donors than that from living donors (22-24). However, we could not find any difference in the expression of TLRs between living and deceased donors. Our data suggest that the TLR pathway is not important for IRI and DGF after transplantation, and that even a low level of TLR expression is sufficient to initiate an immune response.

Alteration of gene expression is the result of inflammatory cell infiltration

Gene expression alterations between paired pre-implantation and acute rejection biopsies were analyzed in 75 patients. The majority of TLRs (TLR1-3 and TLR6-10), C2, and BAX:BCL2 mRNA levels were increased, whereas the expression levels of C4 and the complement regulators (CD46, CD55, and CD59) were decreased at the moment of AR compared to the situation before implantation. The changes in expression levels of TLR4, TLR5, C3, and CR1 varied among the patients with acute rejection. We speculate that the changes in mRNA expression are the result of infiltration of inflammatory cells. Therefore, the correlation between expression level of innate immunity genes and inflammatory markers (CD163, CD68, CD20, and CD3e) at time of AR was analyzed. The expression of TLR1, TLR4, TLR6-10, C2, C3, CR1, and BAX:BCL2 was positively correlated with one or more inflammatory markers, while the expression of CD46 and CD59 was negatively correlated with macrophage markers. In addition, immunohistochemical staining for TLR4, TLR9, and BCL2 confirmed that their expression was relative higher in acute rejection group than in patients with stable graft function. The influx of inflammatory cells can at least partly explain the altered

gene expression between implantation and AR biopsies. However, most of genes expressed during AR show no association with any of the Banff classification scores.

The TLR2 and TLR3 mRNA levels were minimally increased but did not correlate with any inflammatory makers, suggesting that these mRNA levels are dominantly expressed in renal parenchymal tissue and activated by inflammation, which is consistent with a previous study showing a similar TLR3 expression pattern (25). The expression of TLR4 was correlated with myeloid cell markers but the changes in expression varied among patients, suggesting that TLR4 is expressed in both myeloid and parenchymal cells. Indeed, immunohistochemical staining in our study and in other studies showed that TLR4 protein expression could be detected in both endothelial and tubular cells (24, 26-28). Thus, the altered TLR4 expression may depend on the extent of myeloid cell infiltration and kidney cell damage. Semi-quantitative immunohistochemical staining showed that patients with acute rejection have a significantly higher expression of TLR4 than patients with stable graft function. A possible explanation may be that inflammatory cells, especially myeloid cells, express high levels of protein but relative low levels of mRNA.

C3 and CR1 showed similar expression patterns as TLR4. The alteration of C3 and CR1 gene expression, which showed a wide range of expression in both pre-implantation and acute rejection biopsy samples, was further investigated with respect to its association with transplant outcome. Patients with increased and decreased gene expression did not differ with regard to the incidence of steroid resistant rejection and long-term graft outcome. The minor decrease in expression of C4 and CD55 lacked any correlation with inflammatory markers and may be the result of renal tissue damage during acute rejection.

TCMR score provide new dimension for acute rejection assessment

Histologic diagnosis of acute rejection according to Banff classification is poorly reproducible among pathologists and difficult to improve in accuracy (29, 30). This limitation may due to the fact that the principal lesions used for rejection diagnosis are also present in other inflammatory responses such as acute kidney injury (AKI), in combination with subjective interpretation. The recent Banff classification describes criteria of acute TCMR, ABMR, and mixed rejection, and it highlights molecular diagnostic techniques as new tools (31, 32). In chapter 4 we investigated the expression of TCMR related makers in an acute rejection cohort transplanted between 1995 and 2005.

TCMR related markers were mostly expressed in T cells, NK cells, and APCs, as was previously identified using microarray technique (33, 34). The T-score, the average z-score of all TCMR related transcripts, was significantly associated with the interstitial inflammation and tubulitis, but not with intimal arteritis. This is not surprising, since the T-score only reflects the degree of infiltration of inflammatory cells into the graft, and not the localization of the infiltrate. Intimal arteritis, characterized as inflammatory cell beneath the endothelium of arteries, could be induced by ABMR or AKI (35), which may explain

the lack of association with the T-score in the current study. Reeve et al also reported that the isolated v-lesions (intimal arteritis with insufficient infiltration of immune cells) had low TCMR scores (33). Therefore, patients with intimal arteritis with a relatively low T-score should not be categorized as TCMR and they would need to be carefully monitored.

Our studies showed that borderline rejection is reflected by a significantly lower T-score than tubulointerstitial rejection. However, in line with a previous study, several patients with borderline rejection had a relatively high T-score, which should be a reason to reclassify these patients as TCMR (33). Our findings give support to the notion that molecular assessment at time of acute rejection aids in predicting therapy sensitivity.

Risk assessment of steroid resistance: E-score

Steroid resistant rejection is associated with inferior long-term graft outcome (10, 36). Prediction of steroid resistance during acute rejection would open the possibility to treat patients immediately with the optimal immunosuppression and prevent unrepairable nephron damage during the period of steroid therapy. Sarwal et al showed that dense B cells infiltration is correlated with steroid resistant rejection and graft loss (10, 37). However, other studies failed to confirm the correlation between infiltration of B cells into the allograft and the response to steroid therapy (38, 39). In addition, the expression of other inflammatory markers in the graft tissue, including FasL (9), LAG-3, CD25:CD3e ratio (36), metallothioneins (8), granulysin (40), and CD68+ (macrophage) (41, 42), and FoxP3 expression in the urinary sediment (43) have been found to be associated with responsiveness to steroid therapy. In chapter 4 we found that an increased mRNA expression of endothelial-epithelial related genes at the moment of acute rejection predicts the responsiveness to steroid therapy.

Halloran's group established a molecular diagnosis system to identify TCMR and ABMR based on microarray data that was derived from over 700 biopsies (33, 34, 44-48). The transcripts used as ABMR classifiers are mainly expressed in endothelial cells, NK cells, and many of these are induced by INF-γ (47, 48). However, we could not identify any ABMR associated parameters in our studies and could not make any correlation with the E-score. Many patients with steroid-sensitive acute rejection showed comparable E-score as patients, who did not have acute rejection (protocol biopsy).

Endothelial cells line the interior surface of glomeruli and peritubular capillaries and they mediate crucial inflammatory processes. The endothelium-epithelium transcript profile, such as TM4SF18, PGM5, and CD34, which are involved in angiogenesis and biological adhesion, may reflect the integrity of nephron and ability of tissue repair after injury. This may explain why the decreased expression profile is associated with resistance of steroid treatment. In line with our finding, several studies showed that severe intimal arteritis and destruction of microvasculature predict steroid resistance of the rejection (49-51). Therefore, the endothelium-epithelium profiles may provide novel markers to predict steroid resistant rejection.

Univariate logistic regression analysis showed that the E-score is a predictor of steroid resistant rejection. Although the performance of the predictive model was modest (AUC=0.70), it might have a high specificity, representing patients with high expression of endothelium related transcripts who do respond to steroid treatment. In addition, the combination of the E-score and other inflammatory makers may generate a more powerful model for assessment of steroid resistance. This may tested further in future studies.

Prediction of long-term graft survival

Short-term graft survival has increased greatly over the last two decades. The half-life for deceased donors was 6.6 years in 1989 and increased to 8.8 years in 2005, which was driven by improvement of fist year attrition rates. However, the long-term attrition rates have hardly improved (52). Identification of molecular markers, which correlate with long-term graft survival, may allow clinicians to carefully monitor the renal function in high-risk patients and may open the way in the prevention of adverse graft outcome.

Steroid resistant rejection is a risk factor of long-term graft loss (10, 36). The effect of steroid resistant rejection on long-term graft survival was assessed in chapter 4. The patients with steroid resistance showed inferior long-term graft survival compared to patients who showed response to steroid therapy. Steroid resistant rejection is correlated with severe vascular rejection and low endothelium and epithelium expression profiles, suggesting that severe kidney injury during acute rejection results in chronic allograft damage.

In chapter 3 we showed that patients with high TLR4 expression during acute rejection have inferior graft survival compared to patients with low TLR4 expression. TLR4 in the allograft may bind to intracellular ligands released by dead cells, and provide additional proinflammatory signalling to enhance inflammation. Although the antirejection therapy successfully normalized kidney graft function, as reflected by decreased serum creatinine, the high expression of TLR4 may lead to production of higher levels of proinflammatory cytokines and chemokines that induce inflammatory cells infiltration into the allograft after the antirejection therapy and contribute to chronic allograft nephropathy.

The high BAX:BCL2 ratio reflects the high extent of apoptosis and it predicts inferior long-term graft survival in deceased donor groups. On the one hand, apoptosis of parenchymal cells directly leads to the loss of kidney function. However, apoptotic cells attract phagocytic cells into the graft and may be rapidly cleared (53, 54). The accumulated phagocytic cells can be triggered by a danger signal and mediate chronic allograft nephropathy (55). If the apoptotic cells in allograft are not rapidly cleared, they undergo necrosis and release damage-associated molecular patterns (DAMPs) that initiate immune responses (56). Thus, monitoring of the BAX:BCL2 ratio during AR may offer a predictive value with respect to long-term graft survival. Future studies should contain a more in-depth analysis of the presence and kinetics of dying cells in the graft, and their possible impact on outcome.

Genetic risk factors in kidney transplant: lack of validation and small effect

The role of HLA molecules in the transplantation field has been widely recognized: better matching between donor and recipient leads to better graft function. Numerous candidate single nucleotide polymorphisms (SNPs) have found to be associated with occurrence of acute rejection and with outcome, but most studies focused on immune response related genes (57-63). GWAS represent an unbiased approach to simultaneously analyse millions of SNPs and to identify novel makers involved in allograft rejection.

In chapter 5 we performed a GWAS of acute rejection in kidney transplantation. The significant candidate SNPs identified by current GWAS could not be verified in an independent cohort in another transplant center. In line with a previous study, we found that patients with acute rejection show a higher C allele frequency (rs1801274 in FCGR2) compared to the stable graft function group (64). Apart from this specific SNP, most of previously published SNPs could not be confirmed in the current GWAS. Consistent with a well powered GWAS in bone marrow transplantation, the previously reported genetic variants were most likely false positive findings (65). As discussed in chapter 7, the main limitation of GWAS is the requirement of stringent significance thresholds ($P<5\times10-8$). Only SNPs with a big effect on transplant outcome could be captured in our relatively small-sized GWAS. Thus, any false positive findings in our study may result from the small effect of individual genetic variants. Individual SNPs identified by GWAS usually have a small effect by themselves on a complex trait such as acute rejection, explaining only less than 10% of susceptibility to the disease even when all available genetic variants are combined (66). To identify true positive, single SNPs, which have a small effect, and to overcome the issue of validation, the only way is to increase sample size by international collaboration in the field of kidney transplantation.

The role of non-HLA antigens have increasingly been reported in kidney transplantation (67-69). As shown in chapter 6, we found no effect of genomic missense SNP mismatching on kidney transplant outcome. Besides, the mismatch load, reflecting the total amount of mismatching of SNPs in coding sequences between recipient and donor, does not have any effect on AR or long-term graft function. If the mismatch load had any effect, living related transplantations with lower mismatch load should have lower incidence of rejection and longer allograft survival compared to living unrelated transplantations that have higher mismatch load. However, a recent meta-analysis showed there was no difference between living related and unrelated kidney transplantations in acute rejection and graft survival rates (70). A reason for the negative finding may be that the effect of mismatching of missense SNPs is low, under the condition of HLA mismatching and efficient immunosuppressive therapy.

The immune regulatory effect of calcium binding proteins

High expression of S100A9 in kidney biopsies during AR is associated with a beneficial effect on long-term graft survival (71, 72). Most of S100A9+ cells are co-localized with CD68 and HLA-DR, and only one-third of them express CD163, suggesting a distinct macrophage population infiltrating the graft. We found in chapter 8 that S100A9 expression varies greatly among CD14 positive monocytes. Unfortunately we were not able to sort monocytes based on their expression level of S100A9 using SmartFlare RNA detection probes. Cytokine expression profiles between S100A9high and S100A9low subsets were not significantly different. We did find that overexpression of S100A8/A9 in monocyte-derived macrophages leads to increased reactive oxygen species (ROS) production, as well as increased IL-10 mRNA expression (Figure 1). The extracellular ROS may have a negative impact on T cell activation and their subsequent proliferation (73), which may dampen the immune response in the allograft. The consistent increase of IL-10 may represent another anti-inflammatory mediator in such immune response, even though the protein level of IL-10 could not be detected in the supernatant of the transfected cells. We hypothesize that the anti-inflammatory effect of S100A8/A9 proteins may explain their beneficial effect on kidney graft survival.

The THP1 macrophage cell line, which lacks co-stimulation molecules, is unable to stimulate allogeneic T cell activation. The cell viability and stimulation ability of monocyte-derived macrophage were negatively affected, probably as a result of the transfection of a large plasmid (~7000 base pairs), which prevented us from performing mixed lymphocyte cultures. To investigate the effect of S100A8/S100A9 on T cell activation and proliferation, downregulation of S100 proteins in macrophages by transfection of small siRNA constructs may be an alternative approach.

Numerous studies reported that the proinflammatory activity of S100 proteins can be used as biomarker of inflammation, infection, and autoimmune disease (74-80). In contrast, S100A9 was proposed as a novel marker of human monocytic MDSCs, which accumulate in kidney transplant recipients and are able to induce expansion of Treg cells in vitro (81, 82). We found that MDSCs in healthy PBMC express slightly higher levels of S100A9 compared to CD14+HLA-DR+ monocytes, but that S100A9 is not suitable as a specific marker of MDSC since all monocytes positively expressed S100A9. Since MDSCs are detected in higher quantities during inflammatory conditions, S100A9 expression should be tested in MDSCs obtained from kidney transplant recipients. Inhibition of S100A9 in such MDSCs should show whether S100A9 mediates the anti-inflammatory effect of MDSCs.

Conclusions and future perspectives

The results presented in this thesis demonstrate that several molecular and genetic markers are associated with kidney transplant outcome. We showed that a decreased expression profile of endothelial-epithelial cells during AR is associated with resistance to steroid

therapy, suggesting that endothelial cell integrity is involved in the efficacy of antirejection treatment. The elevated TLR4 expression and BAX:BCL2 ratio during AR independently predict inferior long-term graft survival. In addition, the genome-wide association study suggests that genetic risk factors in kidney transplantation confer a small effect and future efforts require large international collaborative studies. Furthermore, the overexpression of S100A8/A9 leads to increased ROS and IL-10 production by macrophages, which may explain the beneficial effect of these S100 molecules on kidney allograft survival.

Molecular assessment of renal biopsies has been established and may add a new dimension to histologic diagnosis. However, biopsy related complications such as bleeding, hematuria, and anuria are not completely eliminated. Assessment of noninvasive material, such as blood and urine, is a promising approach. Identification of rejection specific molecular markers in blood and urine may provide new tools to monitor the immune status of the allograft, and may allow for early detection of subclinical rejection and timely therapeutic intervention.

External validation of potential molecular predictors is necessary in biomarker discovery. The observed prognostic value of the E-score, TLR4, and BAX:BCL2 ratio need to be verified in an independent study cohort. Due to frequently reported false positive findings in genetic association studies, validation by international collaborative studies is highly recommended. In addition, a prospective cohort study is the golden standard to confirm the predictive value of candidate biomarkers before their clinical implication in kidney transplantation.

The potential immune regulatory effect of calcium binding proteins need to be further investigated. The knockdown of S100A8/A9 by transfecting siRNA into macrophages would be an alternative approach to investigate the macrophage stimulation ability by mixed lymphocytes cultures. For characterizing the cytokine expression profile, such as TNFα, IL6, IL10 and TGFβ, concentrated cell culture supernatant may provide valuable information. Thanks to the development of tissue imagine technique for simultaneous analysis of multiple markers by immunohistochemistry, the effect of inflammatory cell subsets infiltrating in the allograft and their possible relationship and interaction in space may be clarified in relation to the clinical outcome of kidney transplant patients.

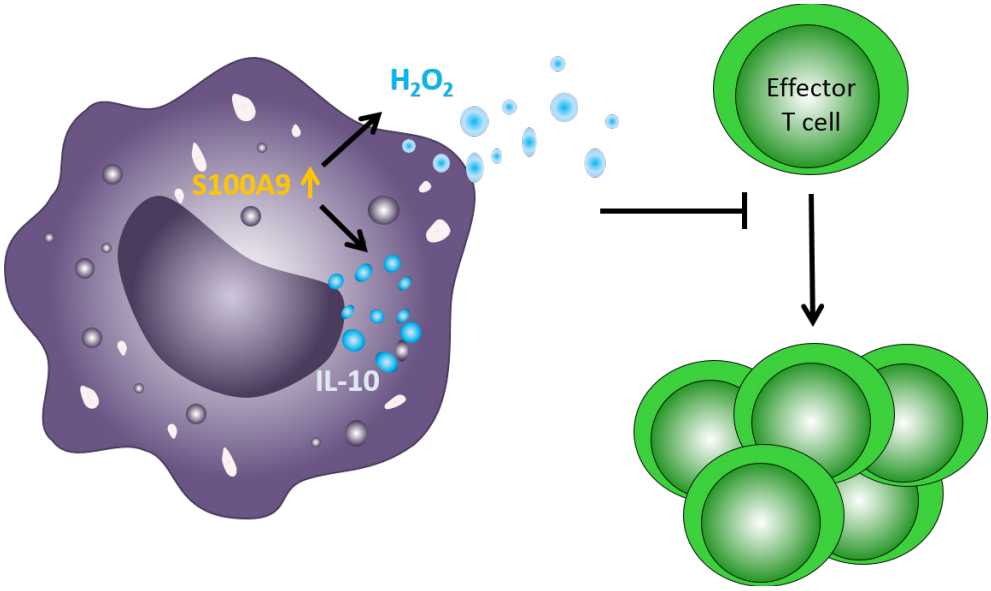


Figure 1. Increased expression of S100A9 in macrophage leads to increased ROS production and elevated IL-10 expression. ROS, reactive oxygen species.

References

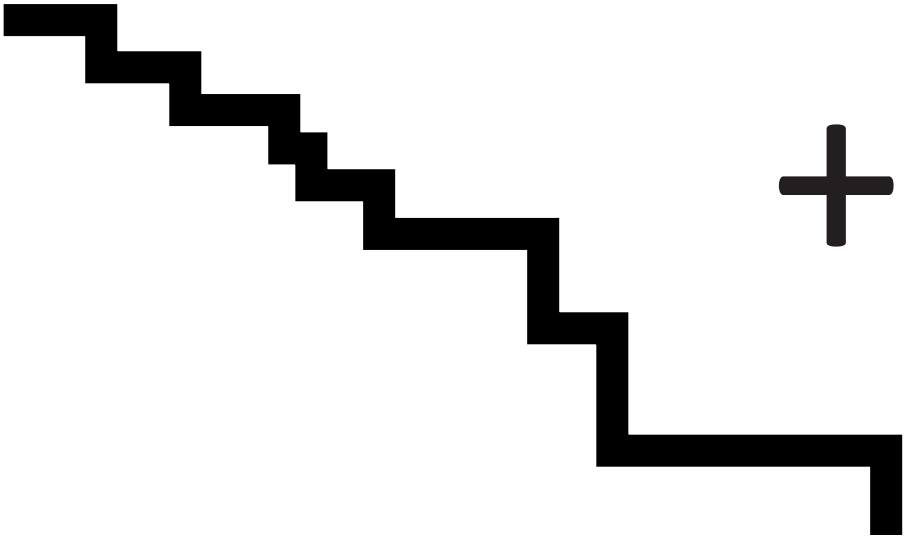
1. Wolfe RA, Ashby VB, Milford EL, Ojo AO, Ettenger RE, Agodoa LY, et al. Comparison of mortality in all patients on dialysis, patients on dialysis awaiting transplantation, and recipients of a first cadaveric transplant. The New England journal of medicine. 1999;341(23):1725-30.
2. Pallardo Mateu LM, Sancho Calabuig A, Capdevila Plaza L, Franco Esteve A. Acute rejection and late renal transplant failure: risk factors and prognosis. Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association. 2004;19 Suppl 3(suppl_3):iii38-42.
3. Pallardo Mateu LM, Sancho Calabuig A, Capdevila Plaza L, Franco Esteve A. Acute rejection and late renal transplant failure: risk factors and prognosis. Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association. 2004;19 Suppl 3(suppl 3):iii38-42.
4. Ojo AO, Wolfe RA, Held PJ, Port FK, Schmouder RL. Delayed Graft Function: Risk Factors and Implications for Renal Allograft Survival1. Transplantation. 1997;63(7):968-74.
5. McLaren AJ, Jassem W, Gray DW, Fuggle SV, Welsh KI, Morris PJ. Delayed graft function: risk factors and the relative effects of early function and acute rejection on long-term survival in cadaveric renal transplantation. Clin Transplant. 1999;13(3):266-72.
6. Gjertson DW. Impact of delayed graft function and acute rejection on kidney graft survival. Clin Transpl. 2000:467-80.
7. 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.
8. Rekers NV, Bajema IM, Mallat MJ, Anholts JD, de Vaal YJ, Zandbergen M, et al. Increased metallothionein expression reflects steroid resistance in renal allograft recipients. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons. 2013;13(8):2106-18.
9. Desvaux D, Schwarzinger M, Pastural M, Baron C, Abtahi M, Berrehar F, et al. Molecular diagnosis of renal-allograft rejection: correlation with histopathologic evaluation and antirejection-therapy resistance. Transplantation. 2004;78(5):647-53.
10. Eikmans M, Roelen DL, Claas FH. Molecular monitoring for rejection and graft outcome in kidney transplantation. Expert Opin Med Diagn. 2008;2(12):1365-79.
11. Li B, Hartono C, Ding R, Sharma VK, Ramaswamy R, Qian B, et al. Noninvasive diagnosis of renal-allograft rejection by measurement of messenger RNA for perforin and granzyme B in urine. The New England journal of medicine. 2001;344(13):947-54.
12. Gwinner W. Renal transplant rejection markers. World J Urol. 2007;25(5):445-55.
13. Suthanthiran M, Schwartz JE, Ding R, Abecassis M, Dadhania D, Samstein B, et al. Urinary-cell mRNA profile and acute cellular rejection in kidney allografts. The New England journal of medicine. 2013;369(1):20-31.
14. Wu WK, Famure O, Li Y, Kim SJ. Delayed graft function and the risk of acute rejection in the modern era of kidney transplantation. Kidney Int. 2015;88(4):851-8.
15. Lechevallier E, Dussol B, Luccioni A, Thirion X, Vacher-Copomat H, Jaber K, et al. Posttransplantation acute tubular necrosis: risk factors and implications for graft survival. Am J Kidney Dis. 1998;32(6):984-91.
16. Patel SJ, Duhart Jr BT, Krauss AG, Moore LW, Egidi MF, Amiri H-S, et al. Risk factors and consequences of delayed graft function in deceased donor renal transplant patients receiving antithymocyte globulin induction. Transplantation. 2008;86(2):313-20.
17. Ounissi M, Cherif M, Abdallah TB, Bacha M, Hedri H, Abderrahim E, et al. Risk factors and consequences of delayed graft function. Saudi J Kidney Dis Transpl. 2013;24(2):243-6.
18. Jung G, Yoon M, Kim S-J, Sin M, Kim E, Moon J, et al., editors. The risk factors of delayed graft function and comparison of clinical outcomes after deceased donor kidney transplantation: single-center study. Transplantation proceedings; 2010: Elsevier.
19. Goncalves-Primo A, Mourao TB, Andrade-Oliveira V, Campos EF, Medina-Pestana JO, Tedesco-Silva H, et al. Investigation of apoptosis-related gene expression levels in preimplantation biopsies as predictors of delayed kidney graft function. Transplantation. 2014;97(12):1260-5.
20. Naesens M, Li L, Ying L, Sansanwal P, Sigdel TK, Hsieh SC, et al. Expression of complement components

differs between kidney allografts from living and deceased donors. Journal of the American Society of Nephrology : JASN. 2009;20(8):1839-51.
21. Wolfs TG, de Vries B, Walter SJ, Peutz-Kootstra CJ, van Heurn LW, Oosterhof GO, et al. Apoptotic cell death is initiated during normothermic ischemia in human kidneys. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons. 2005;5(1):68-75.
22. Leemans JC, Stokman G, Claessen N, Rouschop KM, Teske GJ, Kirschning CJ, et al. Renal-associated TLR2 mediates ischemia/reperfusion injury in the kidney. The Journal of clinical investigation. 2005;115(10):2894-903.
23. Wu H, Chen G, Wyburn KR, Yin J, Bertolino P, Eris JM, et al. TLR4 activation mediates kidney ischemia/reperfusion injury. The Journal of clinical investigation. 2007;117(10):2847-59.
24. Kruger B, Krick S, Dhillon N, Lerner SM, Ames S, Bromberg JS, et al. Donor Toll-like receptor 4 contributes to ischemia and reperfusion injury following human kidney transplantation. Proc Natl Acad Sci U S A. 2009;106(9):3390-5.
25. Dessing MC, Bemelman FJ, Claessen N, Ten Berge IJ, Florquin S, Leemans JC. Intragraft Toll-like receptor profiling in acute renal allograft rejection. Nephrology, dialysis, transplantation : official publication of the European Dialysis and Transplant Association - European Renal Association. 2010;25(12):4087-92.
26. Jang HR, Ko GJ, Wasowska BA, Rabb H. The interaction between ischemia–reperfusion and immune responses in the kidney. Journal of molecular medicine. 2009;87(9):859-64.
27. Samuelsson P, Hang L, Wullt B, Irjala H, Svanborg C. Toll-like receptor 4 expression and cytokine responses in the human urinary tract mucosa. Infect Immun. 2004;72(6):3179-86.
28. Lin M, Yiu WH, Wu HJ, Chan LY, Leung JC, Au WS, et al. Toll-like receptor 4 promotes tubular inflammation in diabetic nephropathy. Journal of the American Society of Nephrology : JASN. 2012;23(1):86-102.
29. Furness PN, Taub N, Assmann KJ, Banfi G, Cosyns J-P, Dorman AM, et al. International variation in histologic grading is large, and persistent feedback does not improve reproducibility. The American journal of surgical pathology. 2003;27(6):805-10.
30. Furness PN, Taub N. International variation in the interpretation of renal transplant biopsies: report of the CERTPAP project1. Kidney international. 2001;60(5):1998-2012.
31. Loupy A, Haas M, Solez K, Racusen L, Glotz D, Seron D, et al. The Banff 2015 Kidney Meeting Report: Current Challenges in Rejection Classification and Prospects for Adopting Molecular Pathology. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons. 2017;17(1):28-41.
32. Haas M, Loupy A, Lefaucheur C, Roufosse C, Glotz D, Seron D, et al. The Banff 2017 Kidney Meeting Report: Revised diagnostic criteria for chronic active T cell–mediated rejection, antibody-mediated rejection, and prospects for integrative endpoints for next-generation clinical trials. American Journal of Transplantation. 2018;18(2):293-307.
33. Reeve J, Sellares J, Mengel M, Sis B, Skene A, Hidalgo L, et al. Molecular diagnosis of T cell-mediated rejection in human kidney transplant biopsies. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons. 2013;13(3):645-55.
34. Venner J, Famulski K, Badr D, Hidalgo L, Chang J, Halloran P. Molecular Landscape of T Cell–Mediated Rejection in Human Kidney Transplants: Prominence of CTLA4 and PD Ligands. American Journal of Transplantation. 2014;14(11):2565-76.
35. Mengel M, Sis B, Haas M, Colvin RB, Halloran PF, Racusen LC, et al. Banff 2011 Meeting report: new concepts in antibody-mediated rejection. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons. 2012;12(3):563-70.
36. Rekers NV, Bajema IM, Mallat MJ, Zuidwijk K, Anholts JD, Goemaere N, et al. Quantitative polymerase chain reaction profiling of immunomarkers in rejecting kidney allografts for predicting response to steroid treatment. Transplantation. 2012;94(6):596-602.
37. Sarwal M, Chua MS, Kambham N, Hsieh SC, Satterwhite T, Masek M, et al. Molecular heterogeneity in acute renal allograft rejection identified by DNA microarray profiling. The New England journal of medicine. 2003;349(2):125-38.
38. Scheepstra C, Bemelman FJ, van der Loos C, Rowshani AT, van Donselaar-Van der Pant KA, Idu MM, et al. B cells in cluster or in a scattered pattern do not correlate with clinical outcome of renal allograft

rejection. Transplantation. 2008;86(6):772-8.
39. Jiang Y, Wang R, Wang H, Huang H, Peng W, Qiu W, et al. The Effect of Histological CD20-Positive B Cell Infiltration in Acute Cellular Rejection on Kidney Transplant Allograft Survival. J Immunol Res. 2016;2016:7473239.
40. Sarwal MM, Jani A, Chang S, Huie P, Wang Z, Salvatierra O, et al. Granulysin expression is a marker for acute rejection and steroid resistance in human renal transplantation. Human Immunology. 2001;62(1):21-31.
41. Ozdemir BH, Demirhan B, Gungen Y. The presence and prognostic importance of glomerular macrophage infiltration in renal allografts. Nephron. 2002;90(4):442-6.
42. Özdemir B, Bilezikci B, Haberal A, Demirhan B, Güngen Y, editors. Histologic evaluation, HLA-DR expression, and macrophage density of renal biopsies in OKT3-treated acute rejection: comparison with steroid response in acute rejection. Transplantation proceedings; 2000: Elsevier.
43. Muthukumar T, Dadhania D, Ding R, Snopkowski C, Naqvi R, Lee JB, et al. Messenger RNA for FOXP3 in the urine of renal-allograft recipients. The New England journal of medicine. 2005;353(22):2342-51.
44. Halloran PF, Reeve J, Akalin E, Aubert O, Bohmig GA, Brennan D, et al. Real time central assessment of kidney transplant indication biopsies by microarrays: the INTERCOMEX study. American Journal of Transplantation. 2017.
45. Desvaux D, Schwarzinger M, Pastural M, Baron C, Abtahi M, Berrehar F, et al. Molecular diagnosis of renal-allograft rejection: correlation with histopathologic evaluation and antirejection-therapy resistance. Transplantation. 2004;78(5):647-53.
46. Halloran P, Pereira A, Chang J, Matas A, Picton M, De Freitas D, et al. Microarray diagnosis of antibody-mediated rejection in kidney transplant biopsies: An international prospective study (INTERCOM). American Journal of Transplantation. 2013;13(11):2865-74.
47. Sellares J, Reeve J, Loupy A, Mengel M, Sis B, Skene A, et al. Molecular Diagnosis of Antibody-Mediated Rejection in Human Kidney Transplants. American Journal of Transplantation. 2013;13(4):971-83.
48. Sis B, Jhangri GS, Bunnag S, Allanach K, Kaplan B, Halloran PF. Endothelial gene expression in kidney transplants with alloantibody indicates antibody-mediated damage despite lack of C4d staining. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons. 2009;9(10):2312-23.
49. Nickeleit V, Vamvakas EC, Pascual M, Poletti BJ, Colvin RB. The prognostic significance of specific arterial lesions in acute renal allograft rejection. Journal of the American Society of Nephrology : JASN. 1998;9(7):1301-8.
50. Haas M, Kraus ES, Samaniego-Picota M, Racusen LC, Ni W, Eustace JA. Acute renal allograft rejection with intimal arteritis: histologic predictors of response to therapy and graft survival. Kidney Int. 2002;61(4):1516-26.
51. Özdemir BH, Demirhan B, Özdemir FN, Dalgiç A, Haberal M. The role of microvascular injury on steroid and OKT3 response in renal allograft rejection. Transplantation. 2004;78(5):734-40.
52. Lamb K, Lodhi S, Meier-Kriesche HU. Long-term renal allograft survival in the United States: A critical reappraisal. American journal of transplantation. 2011;11(3):450-62.
53. Lauber K, Bohn E, Kröber SM, Xiao Y-j, Blumenthal SG, Lindemann RK, et al. Apoptotic cells induce migration of phagocytes via caspase-3-mediated release of a lipid attraction signal. Cell. 2003;113(6):717-30.
54. Gregory CD, Devitt A. The macrophage and the apoptotic cell: an innate immune interaction viewed simplistically? Immunology. 2004;113(1):1-14.
55. Ricardo SD, van Goor H, Eddy AA. Macrophage diversity in renal injury and repair. J Clin Invest. 2008;118(11):3522-30.
56. Kono H, Rock KL. How dying cells alert the immune system to danger. Nature reviews Immunology. 2008;8(4):279-89.
57. Alakulppi NS, Kyllönen LE, Jäntti VT, Matinlauri IH, Partanen J, Salmela KT, et al. Cytokine gene polymorphisms and risks of acute rejection and delayed graft function after kidney transplantation. Transplantation. 2004;78(10):1422-8.
58. Kruger B, Krick S, Dhillon N, Lerner SM, Ames S, Bromberg JS, et al. Donor Toll-like receptor 4 contributes to ischemia and reperfusion injury following human kidney transplantation. Proceedings of the National Academy of Sciences. 2009;106(9):3390-5.
59. Grinyo J, Vanrenterghem Y, Nashan B, Vincenti F, Ekberg H, Lindpaintner K, et al. Association of four

DNA polymorphisms with acute rejection after kidney transplantation. Transplant International. 2008;21(9):879-91.
60. Almoguera B, Shaked A, Keating B. Transplantation genetics: current status and prospects. American Journal of Transplantation. 2014;14(4):764-78.
61. Eikmans M, de Canck I, van der Pol P, Baan CC, Haasnoot GW, Mallat MJ, et al. The functional polymorphism Ala258Ser in the innate receptor gene ficolin-2 in the donor predicts improved renal transplant outcome. Transplantation. 2012;94(5):478-85.
62. Brown KM, Kondeatis E, Vaughan RW, Kon SP, Farmer CK, Taylor JD, et al. Influence of donor C3 allotype on late renal-transplantation outcome. New England Journal of Medicine. 2006;354(19):2014-23.
63. Varagunam M, Yaqoob MM, Döhler B, Opelz G. C3 polymorphisms and allograft outcome in renal transplantation. New England Journal of Medicine. 2009;360(9):874-80.
64. Yuan FF, Watson N, Sullivan JS, Biffin S, Moses J, Geczy AF, et al. Association of Fc gamma receptor IIA polymorphisms with acute renal-allograft rejection. Transplantation. 2004;78(5):766-9.
65. Karaesmen E, Rizvi AA, Preus L, McCarthy PL, Pasquini MC, Onel K, et al. Replication and validation of genetic polymorphisms associated with survival after allogeneic blood or marrow transplant. Blood. 2017:blood-2017-05-784637.
66. Riancho JA. Genome-wide association studies (GWAS) in complex diseases: advantages and limitations. Reumatología Clínica (English Edition). 2012;8(2):56-7.
67. Gratwohl A, Döhler B, Stern M, Opelz G. HY as a minor histocompatibility antigen in kidney transplantation: a retrospective cohort study. The Lancet. 2008;372(9632):49-53.
68. Kim SJ, Gill JS. HY incompatibility predicts short-term outcomes for kidney transplant recipients. Journal of the American Society of Nephrology. 2009;20(9):2025-33.
69. Zhang X, Reinsmoen NL. Impact of Non-Human Leukocyte Antigen-Specific Antibodies in Kidney and Heart Transplantation. Frontiers in immunology. 2017;8:434.
70. Simforoosh N, Shemshaki H, Nadjafi-Semnani M, Sotoudeh M. Living related and living unrelated kidney transplantations: A systematic review and meta-analysis. World journal of transplantation. 2017;7(2):152.
71. Eikmans M, Roos-van Groningen MC, Sijpkens YW, Ehrchen J, Roth J, Baelde HJ, et al. Expression of surfactant protein-C, S100A8, S100A9, and B cell markers in renal allografts: investigation of the prognostic value. Journal of the American Society of Nephrology. 2005;16(12):3771-86.
72. Rekers NV, Bajema IM, Mallat MJ, Petersen B, Anholts JD, Swings GM, et al. Beneficial Immune Effects of Myeloid-Related Proteins in Kidney Transplant Rejection. American journal of transplantation. 2016;16(5):1441-55.
73. Belikov AV, Schraven B, Simeoni L. T cells and reactive oxygen species. Journal of biomedical science. 2015;22(1):85.
74. Kane D, Roth J, Frosch M, Vogl T, Bresnihan B, FitzGerald O. Increased perivascular synovial membrane expression of myeloid-related proteins in psoriatic arthritis. Arthritis & Rheumatology. 2003;48(6):1676-85.
75. Odink K, Cerletti N, Brüggen J, Clerc RG, Tarcsay L, Zwadlo G, et al. Two calcium-binding proteins in infiltrate macrophages of rheumatoid arthritis. Nature. 1987;330(6143):80-2.
76. Ryckman C, Vandal K, Rouleau P, Talbot M, Tessier PA. Proinflammatory activities of S100: proteins S100A8, S100A9, and S100A8/A9 induce neutrophil chemotaxis and adhesion. The Journal of Immunology. 2003;170(6):3233-42.
77. Pechkovsky D, Zalutskaya O, Ivanov G, Misuno N. Calprotectin (MRP8/14 protein complex) release during mycobacterial infection in vitro and in vivo. FEMS Immunology & Medical Microbiology. 2000;29(1):27-33.
78. Frosch M, Strey A, Vogl T, Wulffraat NM, Kuis W, Sunderkötter C, et al. Myeloid-related proteins 8 and 14 are specifically secreted during interaction of phagocytes and activated endothelium and are useful markers for monitoring disease activity in pauciarticular-onset juvenile rheumatoid arthritis. Arthritis & Rheumatology. 2000;43(3):628-37.
79. Vogl T, Tenbrock K, Ludwig S, Leukert N, Ehrhardt C, Van Zoelen MA, et al. Mrp8 and Mrp14 are endogenous activators of Toll-like receptor 4, promoting lethal, endotoxin-induced shock. Nature medicine. 2007;13(9):1042-9.
80. Boyd JH, Kan B, Roberts H, Wang Y, Walley KR. S100A8 and S100A9 mediate endotoxin-induced cardiomyocyte dysfunction via the receptor for advanced glycation end products. Circulation research.

2008;102(10):1239-46.
81. Luan Y, Mosheir E, Menon MC, Wilson D, Woytovich C, Ochando J, et al. Monocytic myeloid-derived suppressor cells accumulate in renal transplant patients and mediate CD4(+) Foxp3(+) Treg expansion. American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons. 2013;13(12):3123-31.
82. Meng F, Chen S, Guo X, Chen Z, Huang X, Lai Y, et al. Clinical significance of myeloid-derived suppressor cells in human renal transplantation with acute T cell-mediated rejection. Inflammation. 2014;37(5):1799-805.



NEDERLANDSE SAMENVATTING

Niertransplantatie is de behandeling bij voorkeur voor patiënten met een eindstadium van nierfalen, aangezien het leidt tot betere overleving en hogere kwaliteit van leven vergeleken met dialyse. Het voorspellen van het optreden van DGF, de respons op de behandeling van acute afstoting en lange-termijn uitkomst na niertransplantatie is moeilijk op basis van enkel de klinische parameters. In het eerste deel van dit proefschrift hebben wij beoogd om moleculaire markers voor steroïd-resistente afstoting en lange-termijn overleving te onderzoeken in biopsieën. In het tweede deel hebben wij ons gericht op genetische markers die gerelateerd zijn aan het optreden van acute afstoting. Expressie van S100 calcium-bindende eiwitten A8 en A9 had in eerdere studies verband met de uitkomst na niertransplantatie. In het laatste gedeelte van dit proefschrift hebben wij aanwijzingen gevonden voor immuun-regulerende effecten van deze S100 eiwitten.

Keuze van SYBR green master mix

Kwantitatieve polymerase ketting reactie (qPCR) is een gevoelige en specifieke techniek om, gebaseerd op SYBR green chemie, genexpressieniveaus te meten. Het type van PCR apparaat en master mix kunnen van invloed zijn op de nauwkeurigheid en betrouwbaarheid van de assay. Drie commerciële PCR mixen (ABI, Bio-Rad, Roche) werden getest voor 79 verschillende primerparen die elk een immuun-gerelateerd transcript detecteren. Wij vonden dat de meeste primersets (n=66, 93%) leidden tot één enkel PCR product, zolang het voorgeschreven PCR protocol per mastermix van de overeenkomstige firma strikt gehanteerd werd. Het gebruik van ABI mix, in vergelijking met de andere mixen, leidde tot lagere Cq-waarde (hoger expressiesignaal) in het cDNA en lagere achtergrondniveaus bij negatieve controle DNA samples. Het type van PCR-apparaat had een kleinere invloed op de resultaten dan de bron van de master mix. Gebaseerd op de data hebben wij besloten om alle transcripten in biopsiemateriaal te meten met de ABI mix op een Viia7 PCR apparaat (ABI).

Relatie tussen pre-transplantaat genexpressie-niveaus en de type van donor

In transplantaties van een nier van een overleden donor was geen van de bestudeerde markers in het pre-transplantaat biopt voorspellend voor het optreden van DGF. Expressie van C2, C3 en Bax:Bcl-2 ratio was hoger in transplantaten van overleden donoren vergeleken met die van levende donoren, wat duidt op een rol van complement en geprogrammeerde celdood (apoptose) bij ischemie-reperfusie schade. Verder bevestigen deze bevindingen resultaten van eerdere studies, dat complement-componenten het hoogst aanwezig zijn in donororganen van overleden donoren en dat apoptose geïnduceerd wordt door normothermische ischemieschade. Remming van complement en apoptose zou daarmee een beschermingsmiddel kunnen zijn tegen de effecten van ischemie-reperfusie schade.

Veranderingen in genexpressie zijn het resultaat van ontstekingsinfiltraat

Veranderingen in genexpressie werden bekeken bij 75 patiënten tussen het moment vóór transplantatie en het moment van acute afstoting na transplantatie. Het merendeel van de bestudeerde markers (TLR-1, -2, -3, -6, -7, -8, -9, -10; C2; Bacl:Bcl2) was toegenomen in expressie ten tijde van acute afstoting, terwijl een aantal markers (C4; CD46, CD55, CD59) een afname in expressie lieten zien. Met immunohistochemie konden we bevestigen dat expressie voor TLR4, TLR9 en BCL2 in het biopt verhoogd is ten tijde van acute afstoting. De veranderingen tijdens acute afstoting zouden het gevolg kunnen zijn van een verhoogde mate van ontstekingsinfiltraat. Inderdaad lieten vele van deze markers voor aangeboren immuniteit een verband zien met de hoeveelheid expressie van één of meerdere van de volgende ontstekingsmarkers: CD3e, CD20, CD68 en CD163.

Risico-inschatting van het optreden van steroïd-resistente acute afstoting: de E-score

Steroïd-resistente acute afstoting heeft vaak een ongunstige lange-termijn uitkomst als gevolg. Een betere voorspelling van steroïd-resistentie van de patiënt bij behandeling van een acute afstoting zou de mogelijkheid kunnen geven om patiënten eerder te behandelen met de juiste medicatie en om onherstelbare nierschade te beperken gedurende de periode van de steroïdentherapie. In hoofdstuk 4 vonden wij dat een toegenomen mRNA expressie van endotheel-epitheel-gerelateerde genen op moment van acute afstoting de respons op de steroïdentherapie voorspelt.

Het endotheel-epitheel transcriptieprofiel (TM4SF18, PGM5, CD34), dat betrokken is bij bloedvatvorming en cel-cel adhesie, is waarschijnlijk een afspiegeling van de kwaliteit van de nefronen en het vermogen van de cellen om weefselschade te herstellen. Deze veronderstelling zou verklaren waarom een verlaagd expressieprofiel juist gezien wordt bij steroïd-resistente afstoting. In dezelfde lijn is aangetoond dat ernstige ontsteking van de wanden van de grote vaten en beschadiging van de microvaatjes in de nier steroïd-resistentie van de afstoting voorspellen. Daarom zouden de endotheel-epitheel-gerelateerde expressieprofielen een nieuwe marker kunnen zijn voor dit type van afstoting.

Voorspelling van lange-termijn transplantaatoverleving

In hoofdstuk 3 vonden we dat patiënten met een hoge TLR4 expressie tijdens acute afstoting een slechtere transplantaatoverleving hebben dan patiënten met een lage TLR4 expressie. TLR4 in het transplantaat kan binden aan intracellulaire liganden die vrijkomen bij celdood, en daarmee een pro-inflammatoir signaal vormen. Dus de hoge TLR4 expressie zou kunnen leiden tot een hogere productie van pro-inflammatoire cytokines en chemokines die ontsteking bevorderen in het transplantaat na de anti-afstoting therapie en daarmee bijdragen aan chronische transplantaatschade.

Een hoge Bax:Bcl2 ratio tijdens acute afstoting voorspelt ongunstige transplantaatuitkomst op de lange termijn en het zou een afspiegeling kunnen zijn van een verhoogde mate van

apoptose in het transplantaat. Apoptose van niercellen kunnen direct leiden tot verlies van nierfunctie. Indien de apoptotische cellen niet tijdig opgeruimd worden, ondergaan ze necrose en scheiden ze schade-gerelateerde moleculaire patronen uit die de immuunrespons verder op gang zetten. Het vervolgen van de Bac:Bcl2 ratio tijdens acute afstoting kan dus van prognostische waarde zijn. Toekomstige studies zouden een diepgaande analyse moeten bevatten van dode en stervende cellen in het transplantaat en hun mogelijke effect op uitkomst.

Genetische risicofactoren bij niertransplantatie: klein effect en gebrek aan validatie

In hoofdstuk 5 hebben wij een genoom-brede associatiestudie (GWAS) bij acute afstoting van niertransplantaten uitgevoerd. Significante SNPs die geïdentificeerd werden in het ene cohort konden niet geverifieerd worden in een tweede, onafhankelijk cohort. Een uitzondering was rs1801274 in het FCGR2 gen: een hogere frequentie van het C-nucleotide voor deze SNP werd gevonden bij patiënten met acute rejectie; en dat in beide cohorten. De voornaamste beperking van GWAS is de noodzaak voor sterke significantie ($P<5\times10^{-8}$) voor een individuele SNP in relatie tot de klinische uitkomst. Individuele SNPs die geïdentificeerd worden met GWAS hebben doorgaans maar een klein effect (<10%) op een complex, multifactorieel fenomeen zoals acute afstoting. Om daadwerkelijk betrouwbaar op zich zelf staande SNPs te identificeren die iets zeggen over uitkomst, is het nodig om de groepsgrootte van het cohort uit te breiden door middel van (inter)nationale samenwerking op het gebied van niertransplantatie.

De rol van non-HLA antigenen bij niertransplantatie wordt met toenemende mate benadrukt. Zoals aangetoond in hoofdstuk 6 vonden wij geen effect van genomische missense SNP mismatching op niertransplantatie-uitkomst. De totale hoeveelheid van gemismatchte SNPs, die binnen coderende genoomsequenties liggen, tussen ontvanger en donor heeft geen effect op klinische uitkomst. Een reden voor de negatieve bevinding kan zijn dat het effect van mismatching van missense SNPs laag is, onder het regime van HLA mismatching en efficiënte immunosuppressieve medicatie.

Het immuunregulerend effect van S100 calcium-bindende eiwitten A8 en A9

Relatief hoge weefselexpressie van S100A8 en S100A9 tijdens acute afstoting is geassocieerd met een gunstig beloop na transplantatie. We vonden in hoofdstuk 8 dat het merendeel van S100A9-positieve cellen ook CD68 en HLA-DR tot expressie brengen, en slechts een klein deel CD163. Dit suggereert dat S100A9 een marker is voor een specifieke macrofaagpopulatie die het transplantaat infiltreert. Verder vonden we dat de mate van S100A9 expressie behoorlijk varieert binnen de CD14+ myeloïde celpopulatie. Deze variatie kon helaas niet worden gekoppeld aan functionaliteit. Wel vonden we dat overexpressie van S100A8/A9 in macrofagen leidt tot een verhoogde productie van reactieve zuurstof radicalen (ROS) en IL-10 mRNA expressie. Het extracellulaire ROS kan een negatieve invloed hebben op activatie

en deling van T cellen, wat leidt tot verlaagd immuunrespons in het transplantaat. IL-10 zou daarbij ontstekingsremmend kunnen zijn. Wij poneren dat het anti-inflammatoire effect van S100A8/A9 eiwitten hun gunstig klinisch effect op transplantaatoverleving verklaart.

Conclusies

De resultaten die gepresenteerd zijn in dit proefschrift laten zien dat verscheidene moleculaire en genetische markers gerelateerd zijn aan niertransplantaat-uitkomst. We hebben laten zien dat een verlaagd expressieprofiel voor endotheel/epitheel cellen tijdens acute afstoting geassocieerd is met ongevoeligheid voor steroïdenbehandeling. De verhoogde TLR4 expressie en Bac:Bcl2 ratio tijdens acute afstoting voorspellen onafhankelijk van elkaar ongunstig beloop van het transplantaat. Voorgaande studies hebben laten zien dat relatief hoge expressie van S100A8 en S100A9 tijdens acute afstoting juist gerelateerd zijn aan een gunstig beloop na transplantatie. In dit proefschrift hebben we laten zien dat de overexpressie van S100A8/A9 in macrofagen leidt tot een hogere productie van ROS en IL-10 door deze cellen. Deze bevinding zou het gunstige klinische effect van de S100 moleculen op niertransplantaat-overleving kunnen verklaren. Tenslotte liet een genoom-brede associatiestudie zien dat individuele genetische puntmutaties in het genoom slechts een minimale bijdrage hebben in het risico voor acute afstoting, en dat toekomstige studies op dit gebied brede (inter)nationale samenwerking vereisen.

CURRICULUM VITAE

Jianxin Yang was born on September 1st 1985 in Zhangjiakou, Hebei, China. In 2006, he started his animal and plant quarantine studies at the Hebei North University. He obtained his bachelor degree in 2010 with a graduation research project on parasite identification at the pathology laboratory from the Animal science and Technology College. He then started his studies on preventive veterinary at Jilin University (JLU). He studied the effect of Raf kinase inhibitor protein (RKIP) on the replication and propagation of Newcastle Disease Virus and completed his master degree in 2013 at the department of veterinary virology of JLU. In November 2013, he started the Ph.D. studies described in this thesis at the department of Immunohaematology and Blood Transfusion (IHB) of the Leiden University Medical Center (LUMC) under the supervision of prof. dr. F.H.J. Claas, and dr. M. Eikmans. During his Ph.D. research he studied molecular and genetic risk factors of kidney transplant outcome. In August 2018, he went back to China and will continue his research in the field of kidney transplantation.

Abbreviations

ABMR	antibody mediated rejection
AP-1	activator protein 1
APAF1	apoptotic protease-activating factor-1
APC	antigen presenting cells
ATG	anti-thymocyte globulin
ATP	adenosine triphosphate
BAX	BCL2-associated X protein
BCL2	B-cell lymphoma 2
BH3	Bcl-2 homology domain 3
c-FLIP	cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein
CAN	chronic allograft nephropathy
Caspases	cysteine-dependent aspartate-directed proteases
CCL2	chemokine (C-C motif) ligand 2
CTLA4	cytotoxic T-lymphocyte-associated protein 4
DAMP	damage-associated molecular patterns
DC	dendritic cells
DGF	delayed graft function
Disc	death inducing signaling complex
DSA	donor specific antibody
dsRNA	double-stranded RNA
ESRD	end-stage renal disease
FasL	First apoptosis signal receptor ligand
GWAS	genome wide association study
HLA	human leukocyte antigen
HMGB1	high-mobility group box-1
HSP	heat shock proteins
IF/TA	interstitial fibrosis and tubular atrophy
IFN-γ	interferon gamma
IL	Interleukin
IRAK	IL-1R-associated kinase
IRI	ischemia and reperfusion injury
IVIG	intravenous immunoglobulin
LAG-3	Lymphocyte Activating 3
LPS	lipopolysaccharide
MAC	membrane-attack complex
MAPK	mitogen-activated protein kinase
MBL	mannan-binding lectin
MDSC	myeloid-derived suppressor cell

MHC	major histocompatibility complex
MYD88	myeloid differentiation primary response protein 88
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK	natural killer
NLR	nucleotide-binding oligomerization domain-like receptors
PAMP	pathogen-associated molecular patterns
PD	programmed death
PGM5	Phosphoglucomutase 5
PRR	pattern recognition receptors
PTC	peritubular capillaries
RAGE	receptor for advanced glycation end products
RIG	retinoic acid–inducible gene I
RLR	RIG-like receptors
ROS	reactive oxygen species
SNP	single nucleotide polymorphisms
ssRNA	single-stranded RNA
TCMR	T cell mediated rejection
TGF-β	transforming growth factor beta
TLR	Toll-like receptor
TM4SF18	Transmembrane 4 L Six Family Member 18
TNFα	tumor necrosis factor alpha
TRAM	TRIF-related adaptor molecules
TRIF	TIR domain-containing adaptor protein inducing IFNβ

LIST OF PUBLICATIONS

Yang J, Kemps-Mols B, Spruyt-Gerritse M, Anholts J, Claas F, Eikmans M. The source of SYBR green master mix determines outcome of nucleic acid amplification reactions. BMC research notes. 2016 Dec;9(1):292.

Yang J, Snijders ML, Haasnoot GW, van Kooten C, Mallat M, de Fijter JW, Clahsen-van Groningen MC, Claas FH, Eikmans M. Elevated intragraft expression of innate immunity and cell death-related markers is a risk factor for adverse graft outcome. Transplant immunology. 2018 Jun;48:39-46.

Yang J, Claas FH, Eikmans M. Genome-wide association studies in kidney transplantation: Advantages and constraints. Transplant immunology. 2018 Aug;49:1-4.

Yang J, Anholts J, Kolbe U, Stegehuis-Kamp J, Claas F, Eikmans M. Calcium-Binding Proteins S100A8 and S100A9: Investigation of Their Immune Regulatory Effect in Myeloid Cells. International journal of molecular sciences. 2018 Jun 21;19(7):1833.

Rekers NV, Flaig TM, Mallat MJ, Spruyt-Gerritse MJ, Zandbergen M, Anholts JD, Bajema IM, Clahsen-van Groningen MC, **Yang J**, de Fijter JW, Claas FH. Donor genotype and intragraft expression of CYP3A5 reflect the response to steroid treatment during acute renal allograft rejection. Transplantation. 2017 Sep 1;101(9).

Acknowledgments

It has been five years since 2 November 2013, the day I walked into the LUMC to start my Ph.D studies at the department of Immunohematology and Blood transfusion (IHB). When I made the decision to go to Netherlands to pursue my PhD, I knew that I would meet many difficulties, such as language problems, a different education background, and many cultural differences. It is great that there were many people willing to help, educate and encourage me to finish my PhD studies and to facilitate my stay in the Netherlands. A few of them I want to thank personally. .

First and foremost, I would like to express my most sincere appreciation to my supervisor Frans, who give me the opportunity to join the ITI group. Frans, you are a great supervisor and leader. You are so smart, knowledgeable, confident and humorous that that I learn a lot from you, not only on scientific work but also on life. Thank you for your trust, guidance, encouragement, and patience throughout these years. This had shaped me both as researcher and a person. I will always be grateful to you. In addition, I especially appreciate you to provide Sissi the opportunity to work in your group. Michael, my daily supervisor and supporter. You were always available for guidance, advice and discussions. Your knowledge and enthusiasm had a big influence on me. You always answered my questions timely and provided me a lot of help, especially with presentations, writing manuscripts and revising my thesis. I have learned a lot from you, for which I am grateful forever. Cees, Dave, Sebas and Leendert, thanks for all the valuable discussions, advice and sharing your knowledge.

I would like to express my appreciation to my roommates, who always supported me, any time and in any way. Jos and Jacqy, my paranymphs, I feel honored that you are standing next to me on this special day. You have helped me from very beginning to the end of my study. Jos, warmhearted colleague and friend, thanks for your guidance on the Dutch culture. Without you, I am afraid, I could not have survived in Holland. Jacqy, like a mom you have taken care of me and shared your fruits with us. Thank you for the enormous support during my experiments especially the ones related to the S100 story. Gonca, my best friend, thanks for your delicious Turkish food, help and guidance. Marijke, thanks for your help in the fluidigm studies. Berit, thanks for your help in the qPCR study. Els Gielis, my closest roommate, thanks for your help in my research and sharing your knowledge. Anita, my last roommate, I really miss the times during which we had a cup of coffee and nice short talks. Thanks for sharing your knowledge especially with respect to the FACS panel.

Anouk, thanks for your secretarial support in the whole process of my stay in the Netherland and my research. Geert, my big supporter in statistics. You are so enthusiastic and always happy to answer any question from me. I really enjoy the discussions with you and have

learned a lot from you. Ellen and Heleen, thanks for take caring of Sissi and me, and for your guidance in cell cultures. Els thanks for your guidance and help in the Luminex experiments. Yvonne de V, Paula, Marry, Yvonne Z, Manon, and Juliette, Thanks for all your help and the pleasant cooperation. The typing and screening lab, thanks all for the pleasant collaboration and necessary support in my research. Godelieve and Janine, Carin and Hanneke, thanks for your kind help and guidance on Immunohistochemistry and FACS staining. Cynthia, Moniek, Caroline and Helena, thanks for all the fun conversations, which I really enjoyed.

I am grateful to our collaborators, Malou. Snijders, Marko Mallat, Johan de Fijter, Marian. Clahsen-van Groningen, Ulrike Kolbe, Geertje. Dreyer, Brendan Keating, Rainer Oberbauer for the valuable comments on my thesis.

All my wonderful friends and family. Dejian Kong, Gangqi Wang, Na Li and Zhuang Li, you were always there, to cook and drink together, to celebrate the Chinese Festival. Thanks for your company and the good times. All the members in the LUMC lunch group, I really enjoy the funny conversations during lunch time.

My parents, it's finally finished. You have seen me grow and listened to my enthusiastic stories from Holland. Thanks for your advice and unconditional support. Dear my wife, you are the greatest supporter. You always take care of me and give your whole love to me. You gave up the opportunity to work and leave your parents to go to Netherlands with me. I really appreciate everything you have done for me and will be forever grateful to you.
In the end, I would like to thank all the people who contributed in any way to my PhD, thank you for the help and support.

Jianxin Yang
杨建新
Dec 2018