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DISSERTATION

Monitoring of tolerance induction and maintenance in clinically relevant transplant models

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1. INTRODUCTION

1.1. Immune response to allogeneic transplants

T cells play crucial roles in initiating destructive immune responses against allogeneic tissue or grafts. In general, T cells can be divided into two major subsets according to their expression of CD4 or CD8 molecules, which recognize specifically distinct foreign antigens that are associated with major histocompatibility complex (MHC) molecules by their T-cell receptor (TCR), CD4 for MHC class II-restricted responses and CD8 for MHC class I-restricted responses (1). Full activation of T cells requires two signals. The first signal conferring specificity to the immune response is the recognition of the TCR-antigen/MHC complex. The second signal (costimulatory signal) is delivered to T cells by antigen-presenting cells (APCs) through the binding of T cell surface receptors such as CD28 to their costimulatory ligands (CD80 or CD86) of the B7 family on the APCs, CD40 to its costimulatory ligand CD154 (CD40L) of TNF-R family on the T cells (**Figure 1**). And increasing numbers of novel costimulatory molecules are been discovered, including inducible costimulatory molecule (ICOS):B7h pathway, programmed death-1 (PD-1):PDL pathway, the CD134:CD134L pathway, the CD27:CD70 pathway (2).

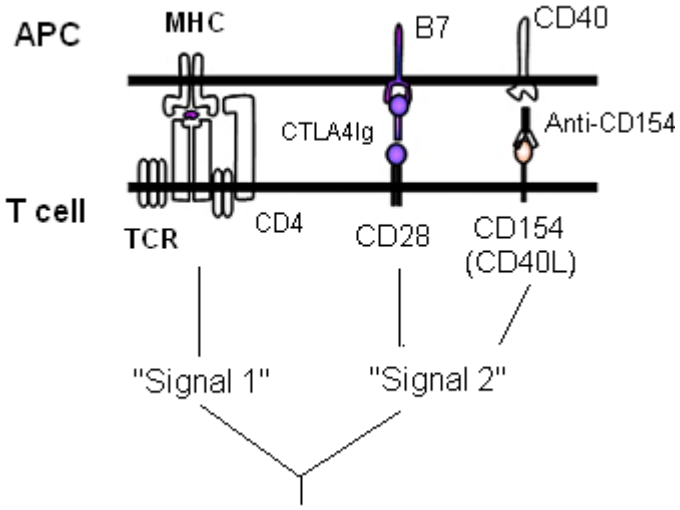


Figure 1. Selected novel strategies for tolerance induction in transplantation

Full activation of T cells by APCs involves two signals. Blockade of one of the signal pathways may induce incomplete alloresponse and tolerance. Modified from Lechler *et al.* (3).

Activated T cells can attack target cells by two approaches. The first cytotoxic T lymphocyte (CTL) effect is mainly accomplished by CD8+ T cells through secretion of perforin and granzyme B, directly lyzing target cells. In addition, the effect can also be mediated by CD4+ T cells through FasL-Fas interactions. Another mechanism is recruitment and activation of the innate immune system mediated by cytokines, which are secreted by T cells, for example, macrophage and eosinophils by secreting interferon-gamma and interleukin-4, respectively. In addition, activated T lymphocytes deliver help for alloantibody production by B cells, enhancing graft damage (3). Clinically, according to timeframe and histopathology the result of the humoral and cellular immune response can be generally classified into different transplant rejection types: hyperacute (minutes to hours), acute (days to months) and chronic rejection (months to years). Hyperacute rejection characterized by thrombotic occlusion of the graft is a complement-mediated cell damage with pre-existing IgM alloantibodies to the donor. Acute rejection is a consequence of T cells and antibodies mediating vascular and parenchymal injury, while chronic rejection is characterized by vasculitis, fibrosis and thrombosis with progressive loss of graft function, namely, chronic allograft vasculopathy (4). Allorecognition triggering cell-mediated rejection is completed by the direct and indirect pathway of antigen presentation (**Figure 2**). The direct pathway is involved in the recognition of intact donor MHC on the surface of donor cells, which predominates in initiating early acute rejection. Indirect pathway is the presentation of processed foreign MHC molecules by recipient APC, dominant in the later stage after transplantation.

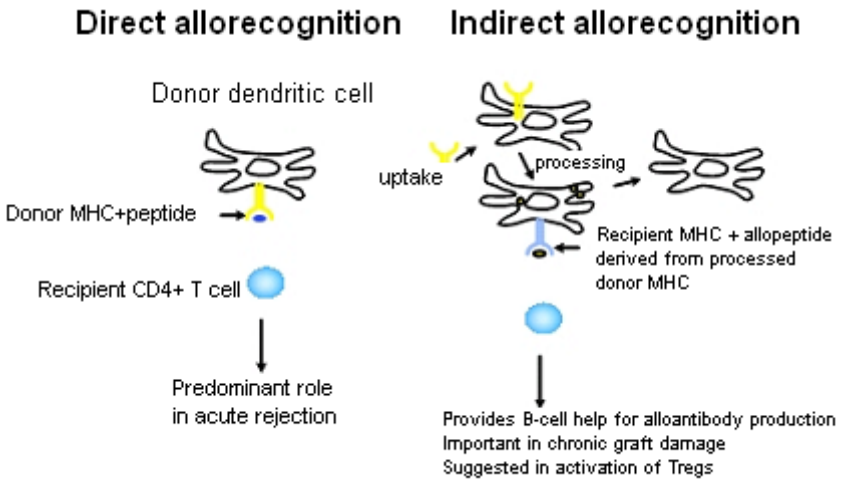


Figure 2. Scheme of two types of allorecognition

During direct allorecognition, donor passenger APCs present intact donor antigens to the recipient T cells, whereas during the indirect pathway of allorecognition, recipient APCs acquire and process donor MHC molecules on the cell surface, which are presented in the context of recipient MHC molecules to the T cells. Source from (3)

1.2. Novel treatment strategies in transplantation

Achieving long-term, drug-free graft acceptance with normal organ function is of enormous importance for the future development of clinical transplantation. Suppression of allograft rejection is still the main focus of modern transplantation medicine. As the understanding of above described immune processes improves, clinically available immunotherapies have been established to prolong allograft survival. Allograft survival has been improved with newer immunosuppressant but no long term graft acceptance is routinely achieved so far and treatment is associated with severe side effects. Currently, a couple of novel compounds and new strategies for transplantation have been described.

Costimulatory blockade. Costimulatory molecules are required for optimal activation of T cells, particularly naive T cells (**Figure 1**). CD28 is a costimulatory receptor expressed primarily by T cells. Its ligands CD80 (B7-1) and CD86 (B7-2) are expressed on the surface of APCs. The soluble receptor-immunoglobulin fusion protein, CTLA4Ig has a higher affinity for B7 family molecules than CD28, and thereby blocks the CD28-B7 signal pathway, successfully prolonging allograft survival in various rodent models (5). Similarly, blockade of the CD154-CD40 signal pathway by using a monoclonal antibody to CD154 can effectively induce tolerance (6). Based upon blockade of these costimulatory molecules, clinical agents have been developed and used in clinical trials (3).

Bone marrow chimerism. This approach is a potent and stable strategy of tolerance induction (TI). The hematopoietic system of the recipient is firstly conditioned by non-lethal total-body irradiation (myeloablative way) or T-cell depleting antibody treatment (nonmyeloablative way), and subsequently reconstituted with allogeneic bone marrow. As a result, donor antigens are exposed to the recipient’s immune compartment, leading to the deletion of potential donor reactive T cells in the thymus. The recipient’s immune system is re-educated to regard allo-antigen as "its self“ (7).

Bone marrow chimerism has been used in clinical pilot trials in which no graft versus host disease (GVHD) or other toxicities were observed (3, 8).

Systemic and oral peptide therapies. Oral administration of a mixture of synthetic class II MHC allopeptides or splenocytes or their lysates can reduce systemic CD4+ T cells and macrophages mediating delayed-type hypersensitivity (DTH) responses *in vivo* and mixed lymphocyte response (MLR) *in vitro* (9, 10). The underlying mechanism probably involves selective inhibition of Th1 cell function and immune deviation to Th2 cell activation (10). A clinical pilot study showed that administration of low-dose donor MHC peptide may effectively inhibit indirect alloreactivity in chronic renal transplant dysfunction (11).

Donor specific transfusion. Experimental data already showed that a single-dose pretransplant infusion of viable donor lymphocytes is capable of inducing long-term allograft survival in humans or animals (12). Based upon dispensable deletion of donor reactive T cells and a significant increase of serum IL-4 level, the suggestive mechanism was the generation of a subset of T cells specifically suppressing antidonor responses in parallel with promotion of IL-4 production in the periphery (12). Additionally, in combination with anti-CD154 mAb, donor specific transfusion of splenocytes could result in improvement of allograft survival in various rodent models (13).

B- or T-cell depletion. An anti-CD20 monoclonal antibody (Rituximab) is specific for the CD20 molecule expressed on the surface of pre-B cells and mature B cells but not plasma cells. Rituximab has been used to successfully treat human steroid-resistant acute cardiac humoral rejection and improved function in highly sensitized kidney transplant patients by depleting B cells and suppressing donor-specific cytotoxic antibodies production (14, 15). A humanized monoclonal antibody Campath-1H (Alemtuzumab) as a powerful anti-lymphocyte antibody directed against surface antigen CD52 expressed on the surface of B- and T- lymphocytes, monocytes and eosinophils has been developed (16). A single dose of Campath-1H can produce a rapid, profound and long-lasting lymphopenia and enable minimization of maintenance immunosuppression due to potent lymphocyte depletion and promotion of peripheral Tregs (regulatory T cells) (17). Campath-1H is used not only

for inducing tolerance in kidney transplantation but also for diminishing the incidence of acute or chronic GVHD (graft versus host disease) (16, 17).

In addition, previous studies showed that depletion of recipient CD4+ not CD8+ T lymphocytes is able to prevent the development of cardiac allograft vasculopathy (18).

Non-depleting anti-T cell antibodies. Tolerance induction can be achieved by non-depleting CD4 and CD8 antibodies across multiple minor or major histocompatibility mismatches in rodent liver, bone marrow, heart, kidney and skin transplant models (19-21). Non-depleting anti-CD4 mAb (RIB5/2) is very powerful in inducing allospecific tolerance by inhibiting the proliferation and cytokine IFN-gamma secretion of alloreactive T cells (22). CD4-targeted therapy with anti-CD4 monoclonal antibody may independently induce transplantation tolerance in sensitized allograft recipients despite persistence of donor-reactive T cells (23). Hence, in our present studies the nondepleting anti-CD4 mAb (RIB5/2) was applied to induce tolerance in rat transplant models.

1.2.1. Effect of non-depleting anti-CD4 antibody (RIB5/2) on graft survival

The CD4 molecule is a 55-kD immunoglobulin superfamily membrane glycoprotein expressed on thymocytes and mature T lymphocytes. During allo-antigen presentation, the extracellular domain of CD4 binds to non-polymorphic regions of MHC-II mainly functioning as a stabilizer of TCR-MHC class II binding, leading to enhanced APC-T cell interaction and T-cell sensitivity to antigen. On the other hand, p56 Lck kinase, a lymphoid-specific cytoplasmic protein tyrosine kinase, which noncovalently associates with cytoplasmic portion of CD4, phosphorylates both CD3-TCR activation complex and ZAP-70 tyrosine kinase, triggering initial events in TCR/CD3 signaling (24). The binding of TCR-CD3 complex delivers the first signal into the T cell (**Figure 3**).

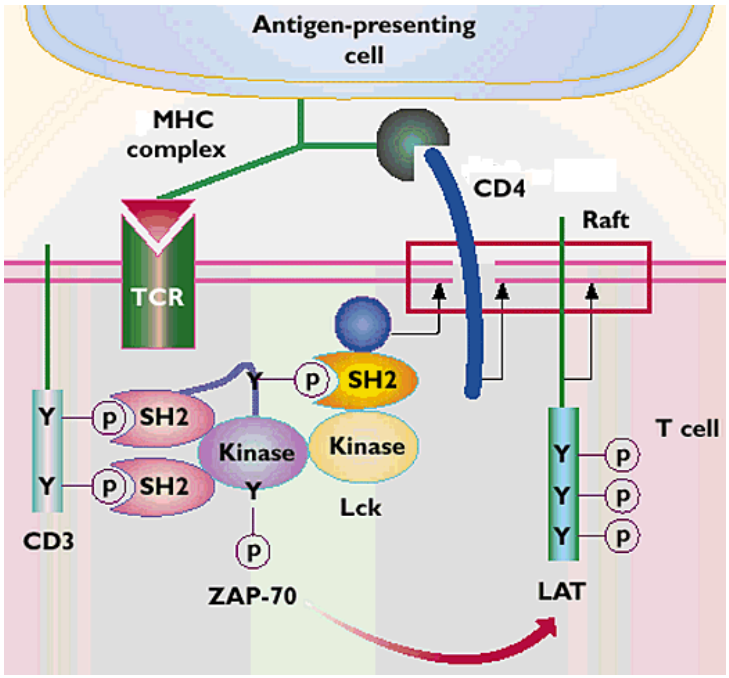


Figure 3. The role of CD4 molecule in APC-T cell interaction

Major histocompatibility complex II simultaneously binds to both a specific TCR and a CD4 coreceptor. The phosphorylation of CD3-TCR complex and ZAP-70 by Lck mediates first signal into the T cell. Modified from Malissen (25).

RIB5/2, a nondepleting anti-rat CD4 monoclonal antibody, is a mouse IgG2a mAb, which targets an epitope on rat CD4+ T cells that is different from those recognized by other binding anti-CD4 mAbs, such as W3/25 or MRC OX35 (26). It has been shown that RIB5/2 modulates the CD4 glycoprotein without eliminating the $CD4^+$ T cells. *In vivo* RIB5/2 treatment in rats resulted in a diminished mean channel fluorescence (MCF) of $CD4^+$ expression without a decrease of the CD4+ stained population by flow cytometric analysis. However, at day 21 post-treatment MCF recovered almost to control levels (27).

A series of studies applying the non-depleting anti-CD4 mAb in different transplant models (MHC-incompatible renal and heart allografts in rat recipients even in high-responder strain combinations and sensitized hosts) demonstrated that RIB5/2 is a powerful inducer of peripheral tolerance. Once generated, the transplant tolerance in recipients induced by anti-CD4 mAb becomes self-sustaining (22, 27).

The mechanism of peripheral tolerance induced by RIB5/2 was investigated in more

detail. *In vivo* and *in vitro*, anti-CD4 mAb treatment completely blocked both IL-2 mRNA expression and protein synthesis by alloreactive T cells but interfered with post-transcriptional regulations that control IFN-gamma production during alloactivation. Further investigations revealed that in anti-CD4-treated T cells the lack of IL-2 production and subsequently dampened activation of the translation initiation factor eIF2alpha account for the dramatically reduced IFN-gamma protein synthesis. It is known that IFN-gamma is an important factor responsible for a protective immunity. In transplantation, excessive production of IFN-gamma leads to graft destruction and eventually graft loss (22).

1.3. Clinical challenges for induction of allograft acceptance

Although transplantation tolerance remarkably progresses both in experimental and in clinical transplantation, there are still a couple of unsolved challenges.

T-cell memory setting. Two distinct processes of T memory generation encompass homeostatic proliferation and heterologous immunity, which have caused a noticeable barrier to tolerance induction and a poor transplant survival. Homeostatic proliferation is also termed lymphopenia-induced proliferation, in which residual T cells proliferate rapidly and differentiate into functional memory T cells under the condition of lymphopenia after alloreactive T-cell depletion. For example, a short course of Campath-1H therapy can effectively induce peripheral T-cell deletion and deplete lymphocytes from both spleen and lymph node. Nevertheless, this deletional approach cannot repress homeostatic proliferation. Moreover, although allograft rejection mediated by naïve T cells can be prevented by $CD4^+CD25^+$ regulatory T cells (Tregs), in either memory CD4 or memory CD8 T-cell-enriched environment Tregs cannot repress the ability of memory T cells to reject the graft (28, 29). Heterologous immunity is a process in which an immune response to infectious pathogens such as viruses can create effector or memory T cells, which may crossreact with other unrelated heterologous viruses or alloantigens, subsequently influencing the course and outcome of unrelated infections or allografts (30, 31). This observation may partially explain why humans and primates are more refractory to tolerance induction than rodents, since the rodents are bred and housed in almost pathogen-free facilities (3). Previous studies demonstrated that rat cytomegalovirus

infection in transplantation can interfere with tolerance induction resulting in chronic allograft damage (30). Additionally, some other factors in clinic such as virus infection can cause not only heterologous immunity but also endogenous IL-2 production, subsequently inducing T cell expansion and IFN-gamma production (32, 33). As a result high IL-2 levels may break the anergy state of alloreactive T cells or abrogate suppression of alloreactive lymphocytes by regulatory T cells (34, 35). Therefore, monitoring the abrogation of tolerance induction by IL-2 is of benefit.

B-cell memory setting. Pathogenic B-cell memory is generated by prior sensitization from previous transplants, pregnancies or blood transfusions. As a result, complement-fixing cytotoxic IgG antibodies are preformed directly against MHC class I specificities, inducing hyperacute rejection with the exception of liver transplant (3). Furthermore, despite low titer or lack of circulating antibodies the presence of memory B cells may still result in acute rejection (36).

Delayed graft function. Delayed graft function (DGF) refers to allograft dysfunction immediately after transplantation, which is a common event at the early stage of kidney transplantation. The earlier identification of DGF would be instrumental in ongoing rescue treatment. By now, a variety of donor- or recipient-dependent factors proved to contribute to DGF including donor age, recipient gender and organ procurement techniques. The occurrence of DGF can lead to an increased incidence of acute rejection (AR) and worse graft outcome (37, 38). The weight of donor and/or recipient as an independent nonimmunologic parameter has also been shown to impact short-, medium- or long-term graft survival in a series of clinical studies (38-42).

1.3.1. Impact of weight difference between donor and recipient on primary graft function

Graft function after transplantation is influenced by donor-dependent and recipient-dependent factors. These donor and recipient nonimmunologic parameters include the quality of the transplanted organ, donor age, donor clearance, recipient gender and the duration of cold ischemia time, influencing short-term and long-term graft survival and correlating with renal function (42, 43).

A series of clinical studies showed that differences in body weight between donor and recipient correlate independently with live-donor graft function, affecting graft function in the short, medium and long-term (39-42). These observations of effects on long-term grafts function were confirmed by accumulating evidences from experimental results (44-46). Indeed, functioning nephron mass of the graft and donor/recipient weight ratios, as a determinant of outcome after kidney transplantation, has been described as important predictors of graft function (47). An insufficient nephron mass might fail to meet the metabolic demands of the kidney recipient resulting in hyperfiltration as a mean of homeostatic adaptation (48). It was shown that hyperfiltration lesions in the small donor kidney due to the high cardiac flow of large recipients exert negative influence on graft function. Hyperfiltration injuries secondary to an inadequate nephron mass are likely to have an additive effect on the damage caused by immune-mediated injuries (39). Sustained nephron hyperfiltration and/or its hemodynamic alterations result in progressive glomerular lesions. A pathological sclerosis in the development of chronic allograft failure cannot eventually be avoided in the glomeruli of the residual nephrons (49).

The intriguing observation was that reduced mass of kidney graft can affect early graft function (41, 48), leading to short-term graft loss rather than long-term graft survival (50). It was previously shown that weight difference between donor and recipient is a surrogate marker of nephron mass loss (51). Body weight of donor or recipient is also associated with early graft function in human kidney transplantation (52, 53). Therefore, studies on how weight difference between donor and recipient impacts early graft function are of extreme importance since the magnitude of early graft dysfunction is closely related with incidence of delayed graft function (54).

At molecular biological level, reduction of functioning nephron mass led to macrophages infiltration and dense expression of macrophage-associated products such as IL-1, IL-6, TNF-alpha, TGF-beta and platelet-derived growth factor (PDGF) (55). Moreover, TNF-alpha and IL-10 production was associated closely with delayed graft function (DGF) (56). Suppression of TNF-alpha by pentoxifylline can inhibit subsequent superoxide anion release from activated neutrophils, considered as an excellent approach to prevent DGF and vascular toxicity of CsA in the early graft period (57). The donor IL-6 polymorphism has been shown to play a role in acute

allograft rejection being a crucial determinant for long-term graft function (58). Nevertheless, the mechanism of how body weight differences between donor and recipient affect early graft function is still undefined. Furthermore there exist no adequate treatment options.

1.3.2. Impact of heterologous immunity (cytomegalovirus infection) on graft function

Human cytomegalovirus (HCMV), a member of the herpesvirus family, is an important opportunistic pathogen and ubiquitous virus, and subject to being transmitted by the transplanted organ or reactivated under the immunosuppressive regimen applied after transplantation. In practice, CMV infection causes a high morbidity and increased mortality in immunocompromised patients (59, 60).

1.3.2.1. Life cycle of cytomegalovirus

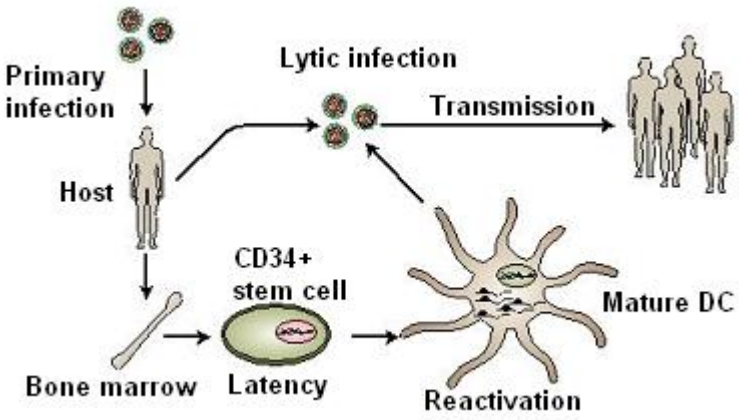


Figure 4. Schematic illustration of the life cycle of human cytomegalovirus

After primary infection, latent HCMV resides in CD34+ bone marrow progenitor cells. As CD34+ cells are differentiating into a mature dendritic cell phenotype, lytic gene expression is reactivated from quiescent genome status, subsequently leading to the release of HCMV virus particles and transmission. Figure taken from (61)

The variation of HCMV prevalence from 50 to 90% is dependent upon the socioeconomic status of the population. HCMV in its latent form is able to persist in

the infected host for a lifetime, with infectious virus particles being detectable.

Although the latent origin of replication of HCMV is unknown, there is HCMV DNA detectable in CD34+ bone-marrow progenitors and their monocyte derivatives (61, 62). Myeloid cells are considered as a critical site for true latency of HCMV in vivo, but other sites of latency cannot be excluded. The mechanism of why the HCMV genome appears selectively in only some cell subpopulations emanating from common CD34+ stem cells that carry viral DNA is obscure. Alternatively, it is possible that HCMV does not replicate in differentiating myeloid cells and in peripheral blood, but resides in bone marrow and is released to peripheral blood. The fact that endothelial cells (ECs) may derive from CD34+ stem cells in the periphery provides the possibility that ECs are a latent site of HCMV, probably contributing to the formation of atherosclerosis. However, no detectable HCMV genomes were found in ECs or vascular smooth-muscle cells from saphenous vein. Macrophages and dendritic cells but not B- or T-cells might be a continual and subclinical reactivation place. CMV can be transmitted via blood products (61, 63). The transmission of HCMV from seropositive blood donors to recipients could be reduced by using leukocyte depleted blood products, suggesting peripheral-blood compartment as one critical viral carrier (62).

Differentiation of CD34+ cells to mature dendritic cell phenotype (Langerhans-like dendritic cells) or of peripheral blood monocytes to monocyte-derived dendritic cells may cause reactivation of HCMV in a natural latency status as chromatin remodeling around the viral major immediate-early (MIE) promoter is dependent on differentiation. The course of HCMV reactivation from latency is of crucial effect on the pathogenesis particularly in immunocompromised individuals (64).

HCMV reactivation from latency is characterized by expression of viral IE (immediate-early) genes. Under an effective immunoresponse of CD8+ lymphocytes, HCMV reactivation will not occur. If primary infection or reactivation of HCMV occurs in an immunocompetent host, clinical disease can be rarely observed. But some infections, allogeneic response, transplant rejection or graft-versus-host disease may stimulate HCMV reactivation in immunocompromised patients (62). Although donor organ may be a source of infectious virus, only transplanting CMV-positive-donor to negative recipients are at highest risk compared to CMV-seropositive recipients. The donor

CMV seropositivity is an independent factor of CMV reactivation (65).

The extremely species-specific nature of HCMV prevents its use in animal models (66). Therefore, rat cytomegalovirus (RCMV) infection in its host has to be utilized as a model for HCMV infection. After acute infection with RCMV (10^5PFU (plaque forming units), i.p. (intraperitoneally)), RCMV can be detected in many organs or peripheral blood at day 4 post infection (67). Subsequently, systemic virus dissemination occurs within 5 to 10 days in all organs, such as liver, kidney, pancreas, lung and spleen as well (68). The IE-AS transcripts of MIE region can be detected in peripheral blood leukocytes (PBLs) from 7 days after inoculation, which indicates amplification of either genomic MIE DNA or unspliced MIE RNA (69). At approximately 8 days post infection, the salivary glands start to produce high levels of infectious virus, considered as a persistent infection. By contrast, no infectious virus can now be detected in other tissues or organs after the first week, however rats are presumed to be latently infected (68, 70).

1.3.2.2. Risks of cytomegalovirus in transplant patients

Cytomegalovirus is one of the most common pathogens after renal transplantation, playing a pivotal role in transplant patients, particularly CMV-associated renal allograft injury. It may affect the transplanted kidneys and recipients by direct and indirect effects. Direct effect of viral infection is viral inclusion in the cells of various tissues, leading to tissue injury and clinical disease. An indirect effect is that low level of viremia and CMV replication causes the host immune system to produce cytokines and chemokines. All these effects increase the incidence of allograft rejection and decrease the survival of recipients (71). An association between acute or chronic rejection and CMV infection has been reported in several studies of renal transplants (60, 72, 73). Thereafter, it was confirmed that viremic CMV infection may independently cause acute tubulointerstitial rejection and acute glomerular injury in kidney allograft recipients, which was concomitantly verified by histologic examination (72, 73).

The mechanism of CMV infection on grafts remains incompletely solved. Investigations at the molecular and cellular level showed that HCMV encodes a

glycoprotein homologous to MHC class-I antigens (74) and can directly enhance MHC class I and intercellular adhesion molecule-1 expression on cultured proximal tubular epithelial cells (PTECs) (75). Immediate-early-2 region of HCMV contains sequence homology to the beta chain of the human histocompatibility complex HLA-DR, which explains why heterologous immunity caused by HCMV infection could contribute to graft rejection by immunologic cross-reactivity after transplantation (76).

A rise of the proportion of CD8+CD69+ but not CD4+CD69+ T lymphocytes in peripheral blood is strongly associated with clinical CMV viremia in patients, which occurs independently of rejection episodes (77). Nonetheless, the relationship between CMV infection and a higher incidence of graft rejection can be explained by the generation of CMV-specific CD8 T cells capable of cross-reacting with alloantigens present on the graft, partially contributing to graft failure (78). The heterologous immunity between exogenous virus and allograft probably exists, which causes crossreactive T-cell responses (79).

1.3.2.3. Cytomegalovirus infection in experimental transplant models

Multiple factors determine the effects of RCMV on allograft outcome in experimental models, such as allospecific immune response, immunosuppression, the timing of RCMV infection and strain combinations.

In experiments, RCMV infection (Maastricht strain, 10^5 PFU, I.p.) could significantly enhance the generation of transplant arteriosclerosis (TA) (80, 81). But an enhancing effect of TA development by RCMV infection was only observed in Lewis recipients, which was also dependent upon the timing of infection. Only during day 1-5 post transplantation, RCMV infection has been shown to cause TA development (82). The mechanism involves an enhanced alloreactivity leading to intensified response of perivascular inflammation after RCMV infection, endothelial cell proliferation, eventual neointima formation and thickening (82).

On the other hand, immunosuppression, alloresponse of the recipient and prolonged cold ischemic time, plays a critical role in the reactivation of RCMV. *In vivo* studies showed that an increased allogeneic response of the recipient by transferring donor leukocytes or eliciting a graft-versus-host reaction may reactivate the latent RCMV in

the recipient (68, 70). Increased immunosuppression and severe graft rejection episodes could cooperatively reactivate CMV while immunosuppression alone was not sufficient (83). Therefore, the combination of immunosuppression and alloresponse is a prerequisite for reactivation of RCMV. Acute CMV reactivation in renal transplant recipients from a latently infected donor is strictly dependent upon the recipient's immunocompetence and the degree of MHC mismatch between donor and recipient (68). Thus, minimizing the allogeneic immune response may prevent the accelerated formation of RCMV-mediated TA after transplantation (82). In addition, prolonged cold ischemic time could synergize with RCMV infection to accelerate injury and dysfunction of kidney graft (84).

With regard to pathogenesis of RCMV at the cellular level, RCMV infection was able to induce an increase of CD4+ cell and macrophage infiltration into the transplanted kidney early post infection, and potentiated glomerular sclerosis and intima proliferation. The underlying mechanism is that RCMV infection led to a significant increase of CD49d-possitive leukocytes into the renal interstitium. CD49d molecule acts as the adhesion receptor VLA-4 on leukocytes, facilitating leukocyte infiltration into kidney allografts (84).

At a molecular level, homologues to MHC class I proteins were also identified in the genomes of either murine or rat cytomegaloviruses (85), which has been speculated as a mechanism for the virus to escape the normal antiviral immune response by the host. Moreover, this result revealed how cytomegalovirus infection contributes to graft rejection after transplantation. In RCMV-infected kidney transplant model, analysis of intragraft gene expression revealed that RCMV caused a significantly prolonged and increased expression of vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) in the vascular endothelium. Furthermore, RCMV infection resulted in elevated numbers of inflammatory cells expressing VCAM-1 and ICAM-1 ligands in the CMV infected grafts compared to noninfected grafts, contributing to accelerated chronic allograft nephropathy (86).

In this study we used the model of RCMV mediated tolerance abrogation to study potential biomarkers which may predict acute and chronic rejection. Early prediction enables early interference therapy such as change of the immunosuppressive treatment and therefore might rescue long-term graft function. Rat cytomegalovirus

infection, resembling closely the human cytomegalovirus, can be used to mimic these clinical challenges.

1.3.3. Impact of inflammation (exogenous IL-2) on graft function

The nondepleting anti-CD4 mAb (RIB5/2) can induce tolerance in MHC-incompatible animal models by completely suppressing IFN-gamma excretion and IL-2 production. Ex vivo and in vivo addition of IL-2 can reverse anti-CD4 mAb mediated T cell unresponsiveness (22). The mechanism is that exogenous IL-2 administration may restore activation of translation initiation factor eIF2alpha and then reactivates the translation of IFN-gamma mRNA. Consequently, excessive IFN-gamma is produced by primed alloreactive T cells in vivo leading to allograft rejection (22, 87).

In clinic some factors such as viral infections can result in enhanced IL-2 production leading to T cell expansion and IFN-gamma production (32, 33). High IL-2 level induced by gamma irradiation may break the anergy status of alloreactive T cells and restore alloresponse of tolerogenic T cells in tolerant host (6, 35).

1.4. Monitoring of transplant outcome

1.4.1. Current monitoring of graft function

Currently, the traditional diagnosis of allograft function is still biopsy, which is an invasive procedure (88). The inevitable complications including haematuria, anuria, perirenal haematoma, bleeding, shock, arteriovenous fistula and even graft loss hamper clinical use for monitoring (89). Inaccessible localization for biopsy examination and sampling errors are additional problems. Moreover, although biopsy diagnosis of renal allograft rejection is usually diagnosed upon the occurrence of graft dysfunction and a mononuclear leukocytic infiltrate, a modest cellular infiltrate is often observed in non-rejecting grafts. Therefore, the examination results are not conclusive.

In addition, serum creatinine is still the gold standard biomarker for the diagnosis of acute rejection. In fact, the rise of serum creatinine is a delayed event, which usually indicates severe and irreversible impairment of kidney graft function. Thus, serum

creatinine is insensitive to mild/moderate decreases in glomerular filtration rate (GFR). Moreover, the rise of serum creatinine does not always correlate with acute rejection, but instead may be associated with cyclosporine-induced nephrotoxicity, pathogenic infections, or other complications. Relevant allograft impairment cannot always be identified effectively and sensitively based on current clinical monitoring techniques (90). Therefore scientists have tried to identify new tools to predict acute rejection and long-term graft function. However, there exist some disadvantages such as false positive results (91) or invasive sampling procedures (92) for patients.

1.4.2. Benefits of non-invasive diagnostic methods for prediction

Novel non-invasive sampling procedures could mean detection of biomarkers in urine or in PBLs (20, 93-95). These surrogate biomarkers would contribute to the quality of life of transplantation patients in many aspects, as they allow frequent monitoring and as they are associated with convenience, no sampling complications, and low cost. The real-time polymerase chain reaction (RT-PCR) technique characterized as a highly sensitive and reproducible molecular approach allows detection of low quantities of as little as 3 pg RNA messenger (mRNA) (96). Many investigators have been using this powerful method to test the diagnostic power of analysing multiple immune activation genes during kidney allograft rejection. Furthermore, its application approved to better understand the molecular mechanisms by using different biologic materials and assessing different markers of immunologic activity (20, 93, 94, 96, 97). Noticeably and importantly, the biomarkers should correspond reliably to the alterations in the allograft itself and should be characterised by high specificity and sensitivity.

1.4.3. Potential markers associated with allograft rejection or tolerance

The surface molecule CD69 is expressed on T lymphocytes and natural killer cells early after their activation. Initially, it has been shown that a heightened frequency CD8+CD69+ T cells in PBLs correlated with acute rejection in renal transplant recipients (98, 99). However, Karpinski M *et al.* demonstrated CD69 expression analysis in PBLs as a noninvasive diagnosis of renal allograft rejection lacks

sensitivity and specificity (77). Cytotoxic lymphocyte gene expression such as perforin, granzyme B, and Fas ligand in peripheral bloods were found to be closely associated with their intragraft expression and a pathologic diagnosis of rejection. In particular, up-regulation of above-mentioned genes in PBLs may serve as a noninvasive method of monitoring renal allograft rejection with excellent positive and negative predictive values (90, 96). Use of anti-rejection therapy resulted in decreases of perforin and granzyme B expression (100). However, infections of cytomegalovirus or BK virus can selectively activate CD8-positive cytotoxic T cells and natural killer cells, causing augmentation of their urinary mRNA expression and misleading clinicians' judgment. Therefore, biomarkers independent of influence of viral infection are critical to predicting graft function.

Work performed by Sawitzki *et al.* resulted in the identification of some genes such as (tolerance associated gene-1) Toag-1 whose transcription is downregulated in the PBLs and corresponding graft samples prior to the rejection, suggesting that these genes may be good markers of an impending immune attack to the allograft that influence graft function and long-term survival (20). Therefore, in the present study, we investigated whether transcriptional analysis of these gene markers by non-invasive method can predict abrogation of anti-CD4-induced tolerance by CMV viral infection or exogenous IL-2.

2. AIMS AND OBJECTIVES

(1) To study the impact of weight difference between donor and recipient on primary allograft function

(2) To study the impact of cytomegalovirus on allograft function

(3) To study the impact of inflammation (exogenous IL-2) on allograft function

(4) To predict long-term allograft outcome under the conditions of inflammation/heterologous immunity

(5) To monitor immunological changes under the conditions of inflammation/heterologous immunity

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Animals

Dark Agouti (DA; $RT1^a$)	Harlan-Winkelmann, Borchen, Germany
Lewis rats (LEW; $RT1^l$)	Harlan-Winkelmann, Borchen, Germany

3.1.2. Reagents, solutions and media

Aquatex	Merck, Darmstadt, Germany
Distilled water	DeltaSelect GmbH, Pfullingen, Germany
Eosin	SIGMA, St. Louis, USA
Ficoll	Pancoll, PAA PAN Biotech GmbH, Germany
4% formalin	Herbeta-Arzneimittel, Berlin, Germany
Harris' Hematoxylin solution	SIGMA, St. Louis, USA
Isoflurane	Forane™, Abbot, Germany
Master Mix	Applied Biosystems, USA
Novaminsulfon	Ratiopharm 1 GmbH, Ulm, Germany
Papanicolaou solution	Merck, Darmstadt, Germany
PBS	GIBCO™, UK
Prednisolone	Slou-Decortin H, Merck, Germany
Streptavidin-horseradish peroxidase conjugate	Peroxidase Complex-DAKO, Denmark
TBS/tween	Merck, Darmstadt, Germany
University of Wisconsin solution	Dupont Pharma, USA

3.1.3. Kits

Absolute RNA Mini-Prep Kit	Stratagene, USA
Agilent RNA 6000 Nano Chip kit	Agilent Technology 2100 Bioanalyzer, USA
QIAmp DNA Blood Mini Kit	QIAGEN GmbH, Hilden, Germany
Stratascript QPCR cDNA Synthesis Kit	Stratagene, USA

3.1.4. Nucleic acids

dNTP's	Amersham, GE Healthcare, UK
Oligo(dT)	Amersham, GE Healthcare, UK
Random primers	Amersham, GE Healthcare, UK

3.1.5. Enzymes

MMLV reverse transcriptase	Promega, USA
DNase	Ambion, Applied Biosystems, USA

3.1.6. Antibodies

Anti-annexin V FITC	BD Pharmingen, Germany
Anti-CD4 mAb, RIB 5/2	EXBIO Praha, Prague, Czech Republic
Anti-ED-1 mAb	Serotec Ltd., Oxford, UK
Anti-Foxp3-PE	eBiosciences, USA
Anti-HO-1 mAb	Stressgen Bioreagents, Canada
Anti-IL-6 mAb	Pierce Biotechnology, USA
Anti-IL6 receptor neutralizing antibody	BIOZOL, Eching, Germany
Anti-Mouse IgG (H+L)-Biotin.rat absorbed	Vector Laboratories, USA
Anti-rat TCR-PerCp	BD Pharmingen, Germany

Anti-rat CD4-FITC	BD Pharmingen, Germany
Anti-rat CD8-APC	BD Pharmingen, Germany
Anti-rat CD45RC-PE	BD Pharmingen, Germany
Goat-anti-Rat-Fab2-IgG-FITC, STAR 69	Serotec Ltd., Oxford, UK

3.2. Rat kidney transplantation

3.2.1. Animals

Naïve male 200-250 g inbred rats (Harlan-Winkelmann, Borchen, Germany) were used throughout the experiments. Lewis rats (LEW; $RT1^{l}$) acted as graft recipients, and Dark Agouti (DA; $RT1^{a}$) animals as donors. The rats were fed with regular rat food and water *ad libitum*. All animal experiments were performed with the permission of the local authorities and according to the German Animal Protection Acts. Anesthesia was induced and maintained with continuous inhalation of O_2 and isoflurane (Forane^{TM}, Abbot, Germany) during whole transplantation procedures.

3.2.2. Donor surgery

The anesthetized donor DA rat was fixed in a supine position. Somatic heparinization was achieved by injection with heparin-Natrium (2000 I.E.) plus 0.6 normal saline via rat dorsal penile vein. A long midline incision was made from the pubic symphysis to the xyphoid. The visceral organs were moved to the right side of the abdomen. The left renal artery and vein were bluntly dissected and separated from each other carefully. The adventitia of kidney was kept well and fat on its surface was almost removed. Abdominal aorta and inferior vena cava were clamped under diaphragm, and inferior vena cava was cut below the left renal vein. The renal allograft was perfused with 20 ml normal saline (4°C) through a 20-gauge catheter (Venflon^{TM}, BD, Germany) and 10 ml University of Wisconsin (UW) solution (4°C) (Dupont Pharma, USA) through 10-gauge catheter (Venflon^{TM}, BD, Germany) placed at the aorta bifurcation until the kidney became uniformly discolored and the perfusate was clear. The ureter was cut close to iliolumbar vein. Donor kidney was stored in UW solution at 4°C for 30-60 minutes.

3.2.3. Recipient surgery

The anesthetized LEW recipient rat was placed in supine position with its tail towards the operator. The left renal vessels were prepared as described above, the ureter was cut close to iliolumbar vein. If there were two renal arteries, the smaller one was ligated with 7-0 silk thread and cut. The renal artery and vein were then clamped with vessel clips next to abdominal aorta and inferior vena cava, respectively. Both renal vessels were cut as near as possible to the kidney. The left native kidney was replaced with the donor allograft. The contralateral kidney was removed immediately or 3 days later, which depended upon detailed groups as are shown below. The ends of renal vessels and the ureters of donor and recipient rats were positioned end-to-end for anastomosis. The graft was covered with cold moisture gauze (4°C) during the period of implantation.

Two stay sutures using 10/0 prolene (Ethicon, USA) were carried out in the opposed ends of the arteries at about 180° to each other. On each side about 3-4 stitches were sutured interruptedly. In order to expose clearly the back wall of the renal artery, vessel clips were reversed up and down.

Two stitches using 10/0 prolene were performed in the opposed ends of the veins as stay sutures at about 150° to each other, leaving a long tail of each suture, thus during suturing veins could be conveniently held. The backside of the vein was initially sutured. The whole circumference of renal vein anastomosis was finished by continuous stitches. The edges of the veins were stretched gently in case of anastomosis constriction.

The ureteral end-to-end reconstruction using 10/0 prolene was performed with 4-6 interrupted stitches. The recipients with postoperative complications such as hydronephrosis and urine leakage were excluded from the study by autopsy.

3.2.4. Reperfusion

As the renal vessel re-construction was established, the clip for the renal vein was released first. Blood returned to the kidney allograft a bit in dark red. Subsequently, the clip for the renal artery was released immediately. A little bleeding occurred, and stopped in a few seconds with cotton swab. Renal artery was clamped again and

more stitches were performed if bleeding was not stopped.

3.2.5. Postoperative care

After the abdomen was closed, the Lewis rat was routinely given 50 mg Novaminsulfon (Ratiopharm 1 GmbH, Ulm) as an analgesic. Infrared light was applied to warm up the rat again.

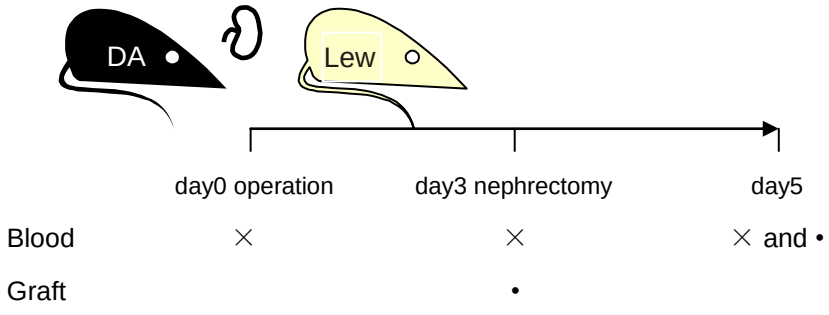
3.3. Experimental groups

3.3.1. Impact of CMV infection on anti-CD4 mAb-induced allograft tolerance

The experimental animals were mainly divided into three big groups—control group (no treatment), tolerance induction group (anit-CD4 mAb treatment) and CMV infection group (anit-CD4 mAb treatment + Maastricht strain or England strain CMV). In subgroups, we have studied the effect of bilateral nephrectomy, prolonged cold ischemia time and different strain CMV infection on allograft outcome (**Table 1**).

In group 1, kidneys were engrafted into unmodified Lewis rats without any treatment (n=6 per time point).

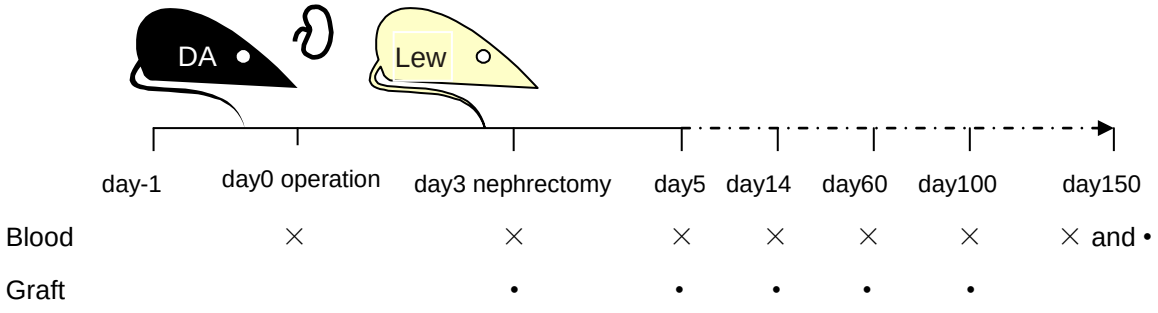
Recipients were sacrificed on day 3 and day 5 post Tx and samples (graft/blood) harvested for further analysis. Additionally, a blood sample was collected on day 0 prior to Tx.



In group 2, Lewis rats received the non-depleting monoclonal anti-CD4 mAb (RIB 5/2) [10 mg/kg body weight; day -1, 0, 1, 2, 3; i.p., n=6-7 per time point].

Grafts were retrieved as indicated below. From the recipients sacrificed on day 150 post-Tx, blood samples were taken serially on days 0, 3, 5, 14, 60, 100 and 150 post-

Tx.



In group 3, Lewis rats were injected intraperitoneally with RIB5/2 (10 mg/kg body weight; day -1, 0, 1, 2, 3; i.p.).

Similar to above. Additionally, the recipients were infected with RCMV 5x10E5 PFU (i.p.) or 2.5x10E6 on day 0 or day –11 and day 0 (n=4-7 per time point).

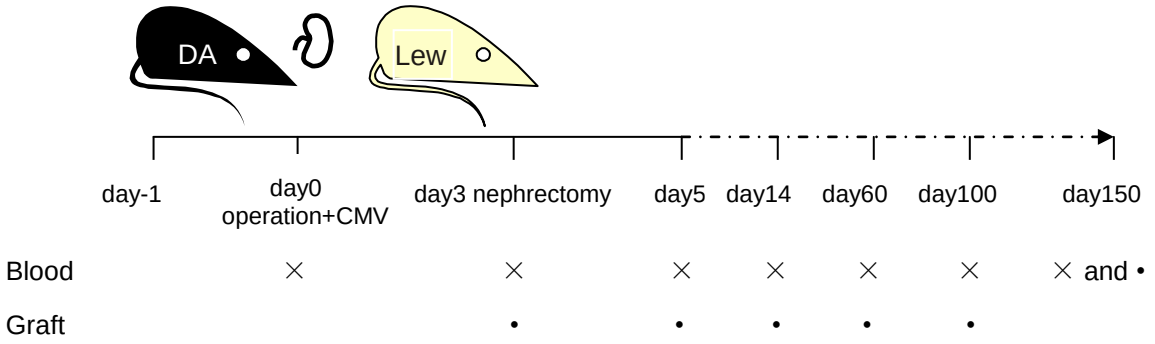


Table 1. Design of experimental sub-groups, treatment characteristics

Group	Sub-group	n	RIB5/2	RCMV	P-CIT§	Harvest (days)	C-N†
Group1	G1	12	No	No	No	3, 5	day 3
	G2	38	Yes	No	No	3, 5, 14, 60, 100, 150	day 3
Group2	G3	7	Yes	No	No	3, 5, 14, 60, 100, 150	day 0
	G4	7	Yes	No	Yes	3, 5, 14, 60, 100, 150	day 0
	G6	37	Yes	5×10E5PFU	No	3, 5, 14, 60, 100, 150	day 3
	G5	7	Yes	5×10E5PFU*	No	3, 5, 14, 60, 100, 150	day 3

Group3	G7	7	Yes	2.5×10E6PFU	No	3, 5, 14, 60, 100, 150	day 3
	G8	4	Yes	2.5×10E6PFU	No	3, 5, 14, 60, 100, 150	day 0
	G9	4	Yes	2.5×10E6PFU	Yes	3, 5, 14, 60, 100, 150	day 0
	G10	6	Yes	Twice CMV infection¶	No	3, 5, 14, 60, 100, 150	day 0

("*" England strain; "¶" RCMV infection at day –14 (2.5×10E6PFU) and at day 0 (5×10E5PFU); "§" prolonged cold ischemia time (P-CIT); "† " contralateral nephrectomy (C-N).)

3.3.2. The effect of weight difference between donor and recipient on primary graft function

This study project was divided into four groups (**Table 2**). The recipients in the G11, G13 and G14 groups received allografts from low-weight donors, designated as high-weight difference (H-WD, -20 to -50% differences in body weight with the percentage reflecting the weight difference in g divided by the weight of the recipient). The recipients of group G12 received kidney grafts from donors demonstrating a similar body weight, designated as low-weight difference (L-WD, -0 to -20% differences in body weight). G13 recipients received in addition a single injection i.v. of 500µg neutralizing anti-IL6 receptor antibody (BIOZOL, Eching, Germany) immediately after transplantation. G14 recipients received a single injection of prednisolone (Slou-Decortin H, 15mg/kg i.v., Merck, Darmstadt, Germany) immediately after transplantation. Both native kidneys were removed at the time of implantation. The animals were sacrificed at 24-h after transplantation. All tissues were snap-frozen in liquid nitrogen and stored at –80°C, or fixed in 4% formalin (Herbeta-Arzneimittel, Berlin, Germany) for haematoxylin/eosin staining.

Table 2. Design of experimental groups with high-weight difference (H-WD) or low-weight difference (L-WD), treatment characteristics

Group	n	Procedure	Species	H-WD	L-WD	anti-IL6R mAb (500µg, i.v.)	Prednisolone (15mg/kg)
G11	6	KTx	DA→LEW	Yes	No	No	No
G12	6	KTx	DA→LEW	No	Yes	No	No
G13	6	KTx	DA→LEW	Yes	No	Yes	No

G14	6	KTx	DA→LEW	Yes	No	No	Yes

3.3.3. The effect of inflammation (exogenous IL-2) on graft function

All recipients received the non-depleting monoclonal anti-CD4 mAb (RIB 5/2) [10 mg/kg body weight; day -1, 0, 1, 2, 3] intraperitoneally (i.p.). Both native kidneys were removed at the time of implantation. This study project was divided into three groups: group 1 (G15), without any further treatment; group 2 (G16) with a low dose of IL-2 (2×10^5 U/day/rat) given i.p. for 10 days starting on day 100 post-Tx; group 3 (G17) with a high dose of IL-2 (8×10^5 U/day/rat) given i.p. for 10 days starting on day 100 post-Tx.

3.4. Estimation of proteinuria and creatinine clearance

Twenty-four-hour urine outputs were collected by placing the recipients individually given free access to food and water in metabolic cages (Harvard Apparatus, Holliston, MA). Creatinine clearance and proteinuria were monitored. Proteinuria was proved to sensitively indicate chronic graft changes in our model (30). Protein excretion (mg/24 h) was measured by precipitation with 20% CCl3COOH. Turbidity was assessed at a wavelength of 415 nm using a Hitachi 911 analyzer (MYCO Instrumentation Source, Inc. Washington, USA).

3.5. Isolation of peripheral blood leukocytes

0.5ml peripheral blood was drawn into EDTA tube from each recipient as described earlier. The blood sample was centrifuged at 3,000rpm at 18°C for 10 minutes. The upper layer of serum was transferred into a new tube and stored at -80°C for subsequent alloantibody analysis. 3 ml lysis buffer (9:1, 0.16M NH_4Cl : 0.17M Trisbase in PBS) was added to the blood cells and incubated at room temperature for 8 minutes. The blood sample was centrifuged at 1,800rpm at 18°C for 10 minutes. The lysis procedure was repeated. The supernatant was discarded and 3 ml sterile PBS was added and the sample vortexed. The sample was centrifuged at 1,800rpm at 18°C for 10 minutes. After the second wash with PBS, the supernatant was

completely removed. The isolated leukocytes were stored at -80°C for subsequent quantitative PCR analysis.

3.6. Quantification of genes by real-time polymerase chain reaction

3.6.1. Sample preparation

The somatic blood of recipient was firstly drained by placing a 20-gauge catheter (Venflon^{TM}, BD, Germany) at the aorta bifurcation until the kidney became discolored. The kidney graft was harvested and longitudinally cut into two main parts. One of them was snap-frozen in liquid nitrogen and the remaining was divided into 4 parts for subsequent histology, immunohistochemistry, PCR analysis.

The renal allograft sample was homogenized with 1.5 ml lysis buffer (containing guanidine thiocyanate)-β-ME mixture using an Ultra-turrax T25 Basic tissue homogenizer (IKA WERKE, Germany).

The appropriate amount of lysis buffer (7µl β-ME added to 1ml Lysis Buffer) mixture (0.4-0.7 ml) was added to peripheral blood leukocytes pellet to ensure that the viscosity of the lysate is low.

3.6.2. Total RNA isolation

Total RNA was extracted from tissue homogenates by using the Absolute RNA Mini-Prep Kit (Stratagene, USA).

3.6.3. Quantitating total RNA

The amount of RNA was quantified by the Agilent RNA 6000 Nano Chip kit (Agilent Technology 2100 Bioanalyzer, USA). 1µl of each RNA sample was pipetted into the indicated well of the RNA chip and compared with 1µl of standard RNA ladder in the same chip according to the manufacturer's instructions.

3.6.4. Reverse transcription

3.6.4.1. Blood samples

6µl RNA (total RNA concentration below 40ng/µl) was used for reverse transcription with Stratascript QPCR cDNA Synthesis Kit (Stratagene, USA) in the total volume of 20µl including 10µl first strand Mastermix (2×), 2µl Oligo dT, 1µl DNase (Ambion), 0.5µl RNase Inhibitor and 1µl Reverse Transcriptase-RNase Inhibitor-Mix. Conditions used for reverse transcription were as follows: 37°C for 30 minutes, 75°C for 5 minutes, 4°C for 2 minutes, 25°C for 5 minutes, 42°C for 45 minutes and 94°C for 5 minutes.

3.6.4.2. Tissue samples

Less than 4 µg extracted RNA (total RNA concentration higher than 40ng/µl) was reverse-transcribed into cDNA using oligo-dT primers and MMLV reverse transcriptase (Promega, USA). The reaction system contained 2µl Oligo(dT), 2µl deoxyribonucleoside (dNTP's), 1µl DNase (Ambion), 8µl RB (5×) buffer, 1µl RNase Inhibitor and 1µl reverse transcriptase. The reaction mixture was adjusted to 40 µl with ddH_2O. Conditions used for reverse transcription were as follows: 75°C for 10 minutes, 37°C for 30 minutes, 75°C for 5 minutes, 42°C for 1 hour and 95°C for 5 minutes.

3.6.5. DNA isolation from whole blood for CMV detection

Peripheral blood sample (0.2-0.3 ml/rat) was collected in a EDTA tube. DNA was isolated using the QIAmp DNA Blood Mini Kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's protocol.

3.6.6. Real-time PCR

3.6.6.1. Principle of TaqMan PCR

The probe used for TaqMan-PCR is labelled with a fluorescence molecule as reporter (6-carboxy-fluorescein, FAM) at 5'- end of oligonucleotide, and a quencher molecule (6-carboxy-tetramethyl-rhodamine, TAMRA) at 3'-end of the same oligonucleotide. This sequence-specific probe hybridizes to an internal sequence of the amplified fragment during the annealing phrase. At this stage quencher and fluorescence molecules are in close proximity (10-100 Å), and the fluorescent emission by the reporter is quenched, and thus not detectable. During PCR extension phase, dual-labelled fluorogenic hybridization probe is cleaved by the 5' to 3' exonuclease activity of the Taq-polymerase, producing an increased fluorescence signal measurable by the Cycler instrument. The fluorescence signal is recorded once at the end of each elongation step.

Gene expression is determined by the increase of fluorescence, corresponding to an exponential amplification and degradation of fluorescent probe. The 'threshold cycle' (C_T value) is defined as fluorescence emission exceeding a manually chosen threshold, the cycle number by which the fluorescence signal exceeds for the first time a chosen background signal, inversely proportional to the original amount of specific cDNA within the sample. To control variation of cDNA concentration in the samples to be compared, a housekeeping gene is utilized as an endogenous control gene. The housekeeping gene used in the present study was rat beta-actin. ΔC_T values for each target gene of specific sample were calculated as follows:

ΔC_T (target gene) = C_T (target gene) - C_T (control gene)

The relative expression was then calculated according to the formula:

Relative expression = $2^{-\Delta Ct}$

3.6.6.2. Performance of Taqman PCR

3.6.6.2.1. RT-PCR of cDNA

The following genes expressions were analyzed for all peripheral blood samples

including CD3, CD25, Foxp3, Perforin, RHAMM, α-Mannosidase, Toag-1, CD40L, CTLA-4. Four additional gene expressions (RhoGAP, CXCL-13, rat IgG, and PDZK-1) were analyzed for graft samples. An overview of all primer set and their corresponding Taqman probes are listed in **Table 3**. The PCR reaction volume was 13µl containing 1µl cDNA, 6.5µl Master Mix (Applied Biosystems, USA), 200nM probe 1µl, 3µl primermix (50 to 900nM of each primer, see table 3) and 1.5µl dH2O. Cycling parameters of TaqMan-PCR were set according to the following conditions: 50°C for 2 minutes for optimal AmpErase UNG activity, 95°C for 10 minutes for activation of the hot-start DNA polymerase and degradation of any preexisting contaminating RNA sequences, and 40 two-stage amplification cycles of 95°C for 15 seconds for denaturation and 60°C for 1 minute for annealing/extension. The TaqMan-PCR was performed using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, USA).

Table 3. List of primers and probes used for Real-Time PCR

Genes	Primers	Sequence (5'-3')	Probe sequence
β-actin	Forward	GTACAACCTCCTTGCAGCTCCT	CGCCACCAGTTCGCCATGGAT
	Reverse	TTGTCGACGACGAGCGC	
TNF-α-intron	Forward	TGAGAGAGTCAGAGCGGTGATTC	ACGTCCCATTGGCTACGAGGTCCG
	Reverse	CCTGCGCCCTCTGGTCTT	
CD3	Forward	CAAAGAAACTAACATGGAGCAGGG	AGGTTTGGCTGGCCTCTTCCTGGTG
	Reverse	CTTTTTGCTGGGCCATGGT	
CD25	Forward	CACAGTCTGTGTACCAGGAGA ACCT	CAGGTCACTGCAGGGAGCCCCC
	Reverse	CCACGAAGTGGTAGATTCTCTTGG	
Foxp3	Forward	AAGTGGCCCGGATGTGAGA	ACTTCCTCAAGCACTGCCAGGCGG
	Reverse	CATTGTGCCCTGCCCTTCT	
Perforin	Forward	GGTGGAGTGGAGGCTTTTGTG	CCAGGCGAAAACTGTACATGCGACACT
	Reverse	CCGAGAAGGCCCATCAGG	
RHAMM	Forward	TGGAAATTAATAAATGGCGTCTCCTA	TTCAGCAACAACTGGATGCCTTTGAAGC
	Reverse	CATTCAACAGTGCCTGCTTCTCT	
RhoGAP	Forward	GGACAGGGTTGCTCCGAGA	TGGCTGAGGCTTGGTAAATCCTGTGCAT
	Reverse	CTGCAACATCAGGAATGGCTG	
α-Mannosidase	Forward	TCTGACCCATGATCCCAAGTACA	TTTCTAGGGCCTCTACGGCTTCCCAGG
	Reverse	CGTCATAACTCTCATGGGCAATG	

Toag-1	Forward	CCCGCCCTCAGAGTCTGAGT	TGATCCTCAGCAGGT ATGCACCAAGCTTG
	Reverse	CCGAGAGGGCTGGGATATTAAA	
CXCL-13		ABI (primer+probe, 0.5µl/13µlPCR)	Custom designed
Rat IgG		ABI (primer+probe, 0.5µl/13µlPCR)	Rn01771406_g1
PDZK-1		ABI (primer+probe, 0.5µl/13µlPCR)	Rn00581820_m1
CD40L		ABI (primer+probe, 0.5µl/13µlPCR)	Rn00584362_m1
CTLA-4		ABI (primer+probe, 0.5µl/13µlPCR)	Rn00581545_m1
TNF-α	Forward	TCGAGTGACAAGCCCGTAGC	CGTCGTAGCAAACCA CCAAGCAGA
	Reverse	CTCAGCCACTCCAGCTGCTC	
IL-6	Forward	AACTCCATCTGCCCTTCAGGA	TTTCTCTCCGCAAGA GACTTCCAGCCA
	Reverse	GGCAGTGGCTGTCAACAACAT	
HO-1	Forward	CAGAAGAGGCTAAGACCGCCTT	TGCTCAACATTGAGC TGTTTGAGGAGCTG
	Reverse	TCTGGTCTTTGTGTTCCTCTGTCA	
BCL-2	Forward	TGA ACCGGCATCTGCACA	AACGGAGGCTGGGAT GCCTTTGTG
	Reverse	CAGAGGTCGCATGCTGGG	
BCL-XL	Forward	GGTGAGTCGGATTGCAAGTTG	CCTGAATGACCACCT AGAGCCTTGGATCC
	Reverse	GTAGAGATCCACAAAAGTGTCCCAG	
BAX	Forward	GCGTGGTTGCCCTCTTCTACTT	CAAACTGGTGCTCAA GGCCCTGTGC
	Reverse	AGCAGCCGCTCACGGAG	
RCMV	Forward	TGCTGACATTCCAATCTCCAAAA	CCATCTCATAATTCGC C
	Reverse	GAACTAACGATGAGCCTTTCGA	

3.6.6.2.2. PCR of DNA for CMV

Primer sequences and corresponding Taqman probes are listed in **Table 3**. PCR reaction conditions were described above.

3.7. Histology and immunohistochemistry

3.7.1. Histology

The harvested specimens were fixed in 4% buffered formalin (Herbeta-Arzneimittel, Berlin, Germany), dehydrated and paraffin-embedded (SHANDON HypercenterXP, England). The tissues were cut into 5µm sections (MICRO HM 400R, Walldorf, Germany), transferred onto slides. Then sections were deparaffinized by 15 minutes

ParaClear (Quartett GmbH, Berlin, Germany) twice, rehydrated through an alcohol series: 100% for 5 minutes, 96%, 80%, 70% for 2 minutes, respectively, rinsed with distilled water (DeltaSelect GmbH, Pfullingen, Germany). Thereafter, Harris' Hematoxylin solution (SIGMA, St. Louis, USA) was applied to stain for 20 minutes, washed with running tap water for 10 minutes. Eosin (SIGMA, St. Louis, USA) was used to counterstain for 2 minutes, rinsed twice with distilled water. The sections were dehydrated through an alcohol series: 80% for 1 minute, 96% for 2 minutes, 100% for 5 minutes, cleared in ParaClear for 30 minutes, assessed by light microscopy.

Allografts harvested from various groups at day 150 post-Tx were used for assessment of glomerusclerosis by Banff mesangial matrix score. The presence of glomerular fibrosis stained by periodic acid Schiff was defined as: grade 1= 0~25%; grade 2=25~50%; grade 3=50~75%; grade 4=75~100% sclerosis of glomerulus. The glomerulosclerosis index = (grade1×glomeruli amount + grade2×glomeruli amount + grade3×glomeruli amount + grade 4×glomeruli amount) / total glomeruli amount.

3.7.2. Immunohistochemistry

The rat specimens were snap-frozen in liquid nitrogen and stored at -80°C. Then the tissues were cut into 5µm sections at -24°C, dried at room temperature (RT) for 2-4 hours, fixed in acetone for 10 minutes at RT and air-dried. The samples were encircled with wax crayon (Pap Pen, DAKO, Denmark) and dried at RT for 30 minutes. After rinsing with TBS/tween (Merck, Darmstadt, Germany) wash buffer twice, the sections were incubated with TBS/Tween/1% BSA/5% horse serum for 60 minutes at RT to block non-specific bindings. Primary antibodies (anti-HO-1 mAb (Stressgen Bioreagents, Canada); anti-IL-6 mAb (Pierce Biotechnology, USA); anti-ED-1 mAb at dilution 1/40 (Serotec Ltd., Oxford, UK)) were used overnight at 4°C in a wet chamber. Then the sections were washed in TBS/tween solution for 5 minutes twice, incubated with secondary antibody (anti-Mouse IgG (H+L)-Biotin.rat absorbed; Vector Laboratories, USA) at dilution 1/75 for 60 minutes at RT in a wet chamber. After washing with TBS/tween and TBS for 5 minutes, respectively, the sections were stained with streptavidin-horseradish peroxidase conjugate (Peroxidase Complex-DAKO, Denmark) for 20 minutes. Subsequently, freshly prepared ABC-complex was

used to incubate for 30 minutes, followed by TBS washing twice. Then the sections were incubated with the substrate chromogen (3-Amino-9-Ethyl-Carbazole, AEC) in the dark for exactly 6 minutes, washed in TBS solution for 5 minutes twice, stained with Papanicolaou solution (Merck, Darmstadt, Germany) for 2 minutes. The slides were then washed in TBS solution and distilled water for 5 minutes twice, respectively and covered with cover glass using Aquatex (Merck, Darmstadt, Germany).

3.8. Flow cytometry analysis

3.8.1. Analysis of alloantibody production

For flow cytometric analysis of circulating allo-antibodies, serum was collected at days 0, 3, 5, 14, 60, 100 and 150 (more samples from days –14 and -9 in CMV twice infection group) after transplantation. Donor DA thymocytes (1×10^6) were incubated with recipients' sera (at dilution 1:10 and 1:100) for 45 min at 4℃. To detect rat IgG in the sera, all samples were incubated with the secondary FITC labelled antibody (Goat-anti-Rat-Fab2-IgG-FITC, STAR 69, Serotec Ltd., Oxford, UK) for 30 min at 4℃. The sera from Lewis rats immunized by DA splenocytes two weeks before were used as a positive control and the obtained signal set to 100%. After washing with FACS buffer, sample cells were fixed with 1% paraformaldehyde and analysed on a FACSCalibur (Becton Dickinson, Germany).

3.8.2. Detection of apoptotic cells

Spleens and 5ml peripheral blood were obtained from untreated kidney graft recipients and anti-CD4 mAb treated tolerance developing kidney graft recipients on day 5 after transplantation. Spleens and peripheral blood from naïve DA rats served as controls. Samples were enriched for leukocytes by Ficoll (Pancoll, PAA PAN Biotech GmbH, Germany) gradient centrifugation (1000g, 20min, RT). Leukocytes were washed twice with Annexin-Binding-Buffer and then incubated with anti-rat TCR-PerCp, anti-rat CD8-APC, anti-rat CD45RC-PE and annexin V FITC for 20min at 4°C. All reagents were obtained from BD Pharmingen (Germany). The samples were run on a FACS Calibur (Becton Dickinson, SanDiego, USA) and analysed using

CellQuest (Becton Dickinson, SanDiego, USA).

3.8.3. Frequency analysis of Foxp3 expressing cells

5ml peripheral blood was obtained from untreated kidney graft recipients and anti-CD4 mAb treated tolerance developing kidney graft recipients on day 150 after transplantation. Samples were enriched for leukocytes by Ficoll (Pancoll, PAA PAN Biotech GmbH, Germany) gradient centrifugation (1000g, 20min, RT) Leukocytes were washed twice with PBS and then incubated with anti-rat TCR-PerCp and anti-rat CD4-FITC (both obtained from BD Pharmingen, Germany) for 20min at 4°C. After an additional washing step with PBS, samples were intracellularly stained with anti-Foxp3-PE (eBiosciences, USA) according to the manufacturer's instruction. The samples were run on a FACSCalibur (Becton Dickinson, SanDiego, USA) and analysed using CellQuest (Becton Dickinson, SanDiego, USA).

3.9. Statistical analysis

Data were analyzed using the statistical software SPSS (SPSS GmbHSoftware, Germany). Data for gene expression between treatment groups were analyzed by using the Mann-Whitney U-test. Statistical difference was accepted as $p<0.05$.

4. RESULTS

4.1. Impact of weight difference between donor and recipient on early graft function

4.1.1. Correlation between weight difference and early graft function

First, we investigated whether differences between donor and recipient body weight can affect early graft function after kidney transplantation. Indeed, as shown in figure 5, body weight differences between donor and recipient is inversely correlated with serum creatinine levels at 24-h post-transplantation (Pearson correlation -0.752, p=0.008). Thus, transplantation of kidney allografts from donors with a reduced body weight, in relation to the recipients, leads to impaired early graft function.

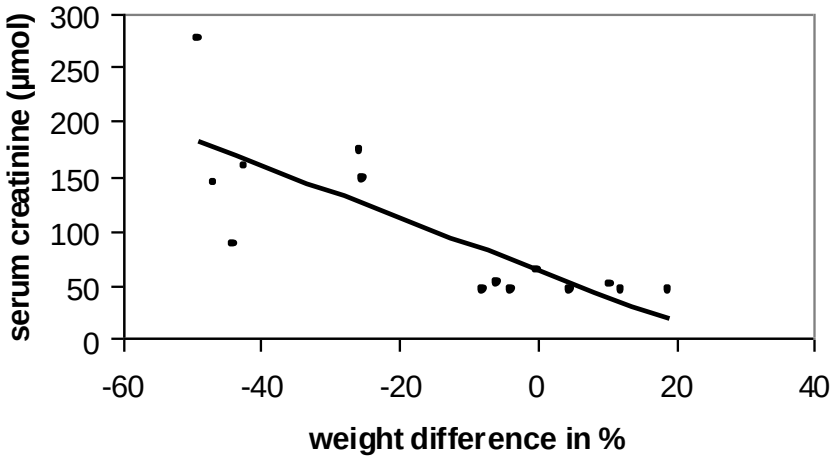


Figure 5. Serum creatinine levels at 24 hours after transplantation inversely correlate with body weight differences.

DA (RT1av1) donor kidneys were transplanted into LEW (RT1) recipients. Recipients received kidney grafts from donors with varying body weight differences (-50% to +20% body weight difference). The serum creatinine levels were determined at 24-h post-Tx. The levels of serum creatinine of recipients with higher weight differences increased dramatically. The alteration of serum creatinine value correlates with increasing body weight differences between donor and recipients (Pearson correlation -0.752, p=0.008).

4.1.2. Intragraft gene expression of inflammatory and apoptosis mediators

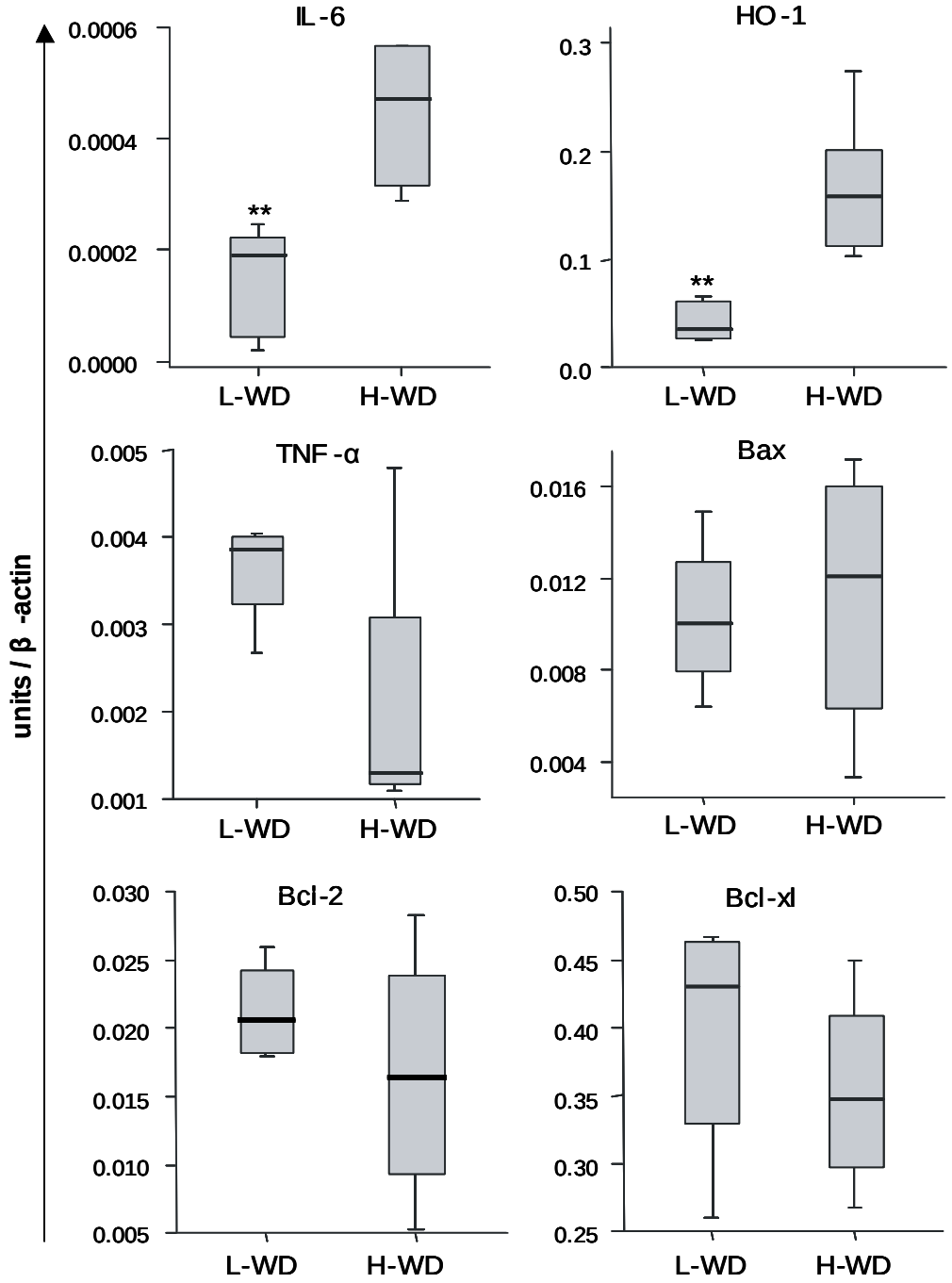


Figure 6. Comparative intragraft gene expression analysis after kidney transplantation

DA (RT1av1) donor kidneys were transplanted into LEW (RT1) recipients. Recipients received kidney grafts from donors with varying body weight differences (L-WD = -20 to +20%; H-WD = -50 to -20% weight difference). Gene expression in allografts harvested on day 1 post-Tx was analyzed by RT-PCR. Results are shown as mean±SD. (IL-6: H-WD vs. L-WD p=0.004, HO-1: H-WD vs. L-WD p=0.004)

In order to determine the molecular mechanisms underlying the impaired early graft function with increasing differences in body weight, we studied the gene expression of several inflammatory and apoptosis mediators. Therefore recipients were divided into two groups. G11 recipients received allografts from low-weight donors, designated as high-weight difference (n=6, H-WD, -20 to -50% differences in body weight with the percentage reflecting the weight difference in gram divided by the weight of the recipient). G12 recipients received allografts from donors with a similar body weight (n=6, L-WD, -0 to -20% differences in body weight). 24 hours after transplantation grafts were harvested and their gene expressions studied applying quantitative RT-PCR. We detected significantly increased HO-1 and IL-6 mRNA expression levels in allografts from H-WD recipients in comparison to those in allografts from L-WD recipients (**Figure 6**, HO-1: p=0.004, IL-6: p=0.004). In contrast, no differences in gene expression of TNF-α and the apoptosis mediators Bcl-2, Bcl-xl, or Bax could be detected (p=0.1905, p=0.5556, p=0.7302, p=0.5556 respectively).

4.1.3. Immunohistochemical analysis of HO-1 and interleukin 6

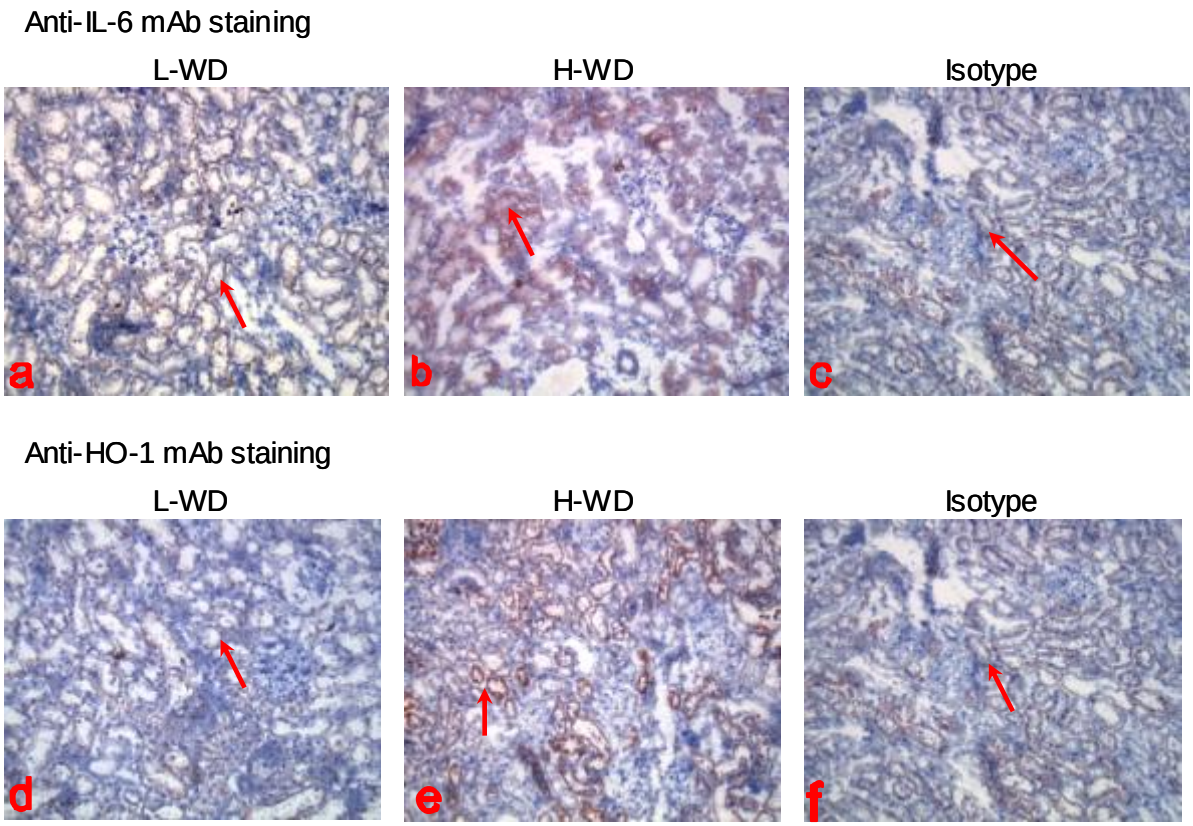


Figure 7. Immunohistochemical analysis of IL-6 and HO-1.

Allografts harvested from L-WD and H-WD recipients on day 1 post-Tx were snap frozen into Tissue Tec and stained for IL-6 and HO-1 protein as described in Materials and Methods. The tubular epithelial cells are indicated with arrows. (x 400 of original magnification).

To investigate which cells within the kidney grafts are responsible for the increased HO-1 and IL-6 expression, immunohistochemical analysis was performed. Interestingly, anti-HO-1 and anti-IL6 staining was more intense in allografts from H-WD recipients (**Figure 7**). Furthermore, immunohistologic staining revealed that HO-1 and IL-6 staining was exclusively localized within tubuli, no staining in glomeruli was detectable, indicating that tubular epithelial cells respond to a weight difference dependent metabolic stress by up-regulating the stress response gene HO-1 and the inflammatory mediator interleukin 6.

4.1.4. Targeting interleukin 6 signaling can rescue primary graft function

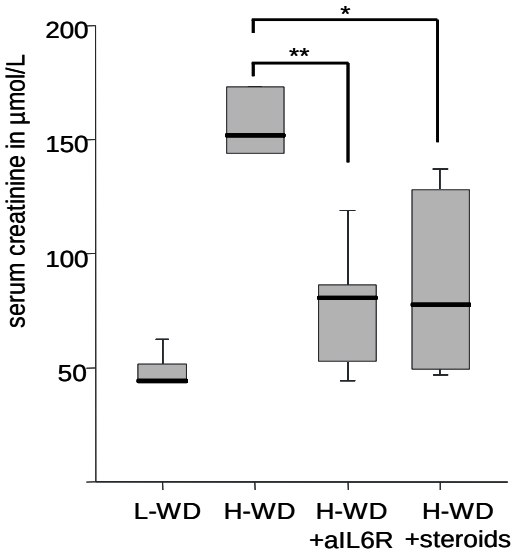


Figure 8. Anti-IL6R mAb and steroid treatment can rescue primary graft function in H-WD recipients.

DA (RT1av1) donor kidneys were transplanted into LEW (RT1) recipients. Recipients received kidney grafts from donors with low-weight differences (L-WD), with high-weight differences left untreated (H-WD), with high-weight differences treated with anti-IL6R mAb (H-WD+aIL6R) or high-weight differences treated with Prednisolone (H-WD+steroids). The serum creatinine levels were determined at 24-h post-Tx. Results are shown as mean±SD. (H-WD vs. H-WD+aIL6R p=0.008, H-WD vs. H-WD+steroids p=0.016).

If the increased interleukin 6 expression by tubular epithelial cells is in part responsible for the impaired early graft function in H-WD recipients, neutralization of interleukin 6 signaling should restore early graft function. In order to test that hypothesis, H-WD recipients received a single injection of a neutralizing anti-interleukin 6 receptor antibody immediately after transplantation (group G13). The ability of interleukin 6 neutralization to restore early graft function was compared to that of a standard unspecific steroid treatment (group G14). Indeed, administration of an anti-IL6 receptor antibody could nearly completely restore early graft function as reflected by a significantly reduced serum creatinine level 24 hours post-transplantation (**Figure 8**, 82.57±30.63µmol/L, p=0.008). The effect was comparable to a steroid treatment of H-WD recipients (86.03±41.44µmol/L, p=0.016).

4.1.5. Neutralization of interleukin 6 signaling prevents tubular damage in H-WD recipients

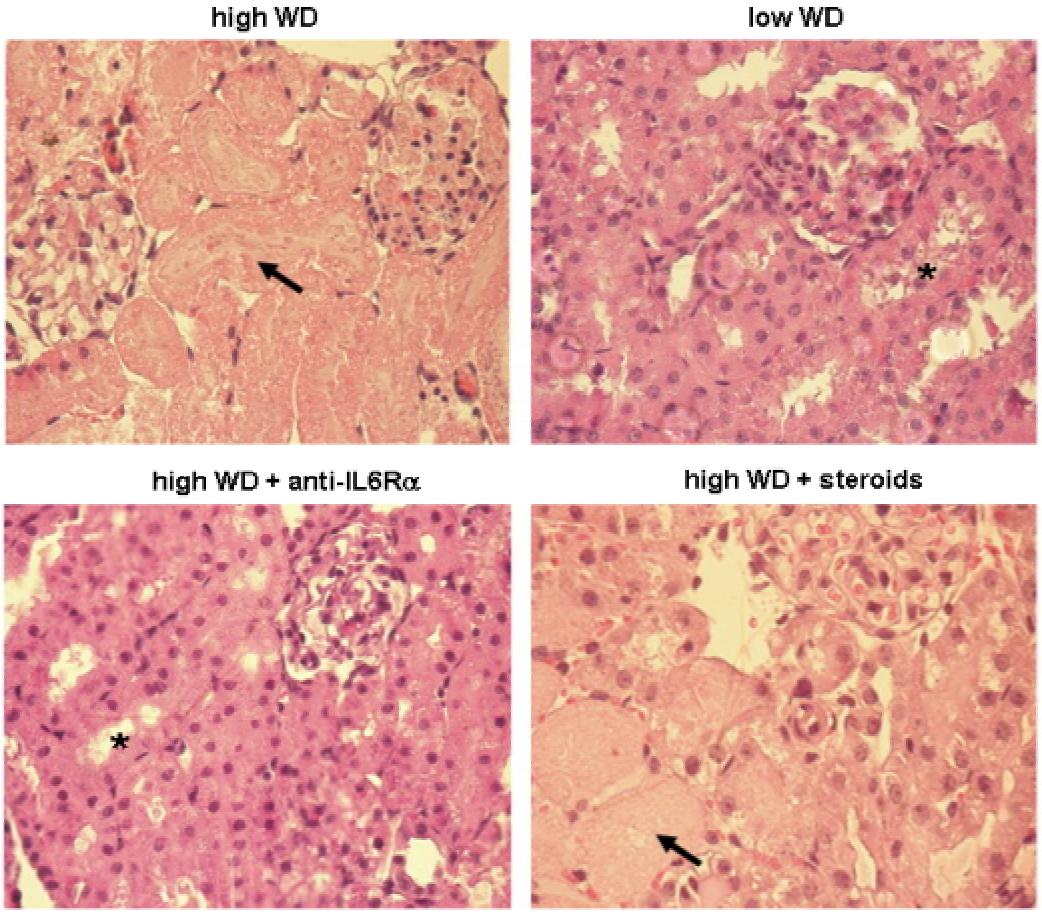


Figure 9. Anti-IL6R mAb treatment can prevent tubuli destruction in H-WD recipients.

Allografts harvested from L-WD, H-WD, H-WD+anti-IL6R and H-WD+steroids recipients on day 1 post-Tx were embedded in paraffin and stained with hematoxylin and eosin as described in the Materials and Methods. High WD kidneys and kidneys from steroid treated H-WD recipients showed a marked degree of tubular injury, including tubular necrosis and dilatation, cast formation, cytoplasmic swelling and nuclear condensation (arrows), whereas low WD kidneys and grafts from anti-IL6R mAb treated recipients were characterized by a well preserved tubular architecture (asterisks).

Unlike allografts from L-WD recipients with relatively preserved tubular histology, allografts from H-WD recipients demonstrated typical features of severe acute tubular injury (**Figure 9**). These features included tubular necrosis and dilatation, cast formation, cytoplasmic swelling, nuclear condensation and complete loss of nuclei in up to 50% of the tubuli. Interestingly, neutralization of interleukin 6 signalling by applying an anti-IL6R antibody restored the tubular morphology indicating a major role of interleukin 6 in the development of the pathological alterations described above. In contrast, a standard steroid treatment could not completely prevent tubular damage in H-WD recipients.

4.2. Impact of cytomegalovirus on tolerance induction

To study the effect of cytomegalovirus on tolerance induction, the recipients were additionally treated with RCMV at a low dose (5x10E5PFU) (G6), a high dose (2.5x10E6PUF) (G7), a high dose plus contralateral nephrectomy on day 0 (G8), a high dose plus prolonged cold ischemic time and contralateral nephrectomy on day 0 (G9), and twice RCMV infection plus contralateral nephrectomy on day 0 (G10).

4.2.1. Detection of CMV copies

After acute infection, RCMV appears in peripheral blood at day 4 (67). Subsequently, systemic virus dissemination occurs within 5 to 10 days in all organs (68). Therefore, RCMV copies might be detected at day 5 in peripheral blood of transplanted recipients.

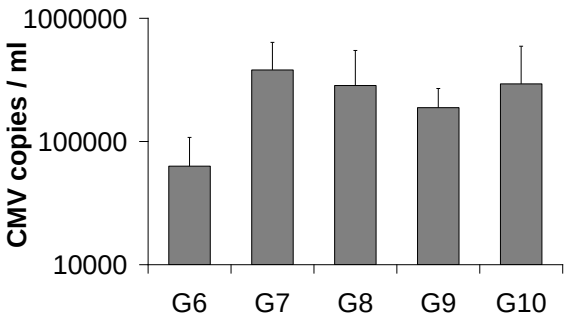


Figure 10. RCMV copies in peripheral blood samples on day 5 after transplantation

Allogeneic kidney transplantation of DA (RT1a) donor kidneys into LEW (RT1*l*) recipients was performed as described in Materials and Methods. Recipients of different groups (G6-G10) were infected by Maastricht strain or England strain RCMV. RCMV copies in peripheral blood were detected on day 5 post-transplantation.

Indeed we detected CMV copies in peripheral blood on day 5 after Tx in all CMV infected recipients (G6-G10) (**Figure 10**). Moreover, a lower amount of CMV copies was found in the low-dose RCMV infected recipients (G6) whereas the recipients infected by high-dose RCMV presented a higher load of CMV (G7, G8, G9 and G10). No CMV copies were found in the anti-CD4 induction group or untreated control group.

4.2.2. Graft function and survival

Currently, serum creatinine is still a traditional marker of glomerular filtrate rate of kidney. Impaired graft function will result in an increase of serum creatinine.

Graft function: In our present study, the untreated LEW recipients of DA grafts (G1) developed impaired graft function and uremia due to acute rejection, as evidenced by steep rise in serum creatinine from day 3 to day 5 post-Tx in comparison to that of recipients in other groups (G1-G10 on day 5: p=0.0007). In the early period post-Tx, Maastricht strain R-CMV at a high dose and when applied twice caused acute rejection, manifested by a sudden rise of serum creatinine values at day 8 and at day 14, respectively. Regardless of kind of CMV infection or alteration of surgical conditions, long-term surviving grafts functioned normally during the late observation period except one recipient in the anti-CD4mAb only group (G2) which experienced

chronic rejection, leading to an increase of serum creatinine on day 140 (**Figure 11, A**). 24-h urine protein levels in the groups of prolonged cold ischemia time (G4), Maastricht strain R-CMV infected at high-dose (G7) and twice (G10) increased on day 150 post-transplantation (**Figure 11, B**).

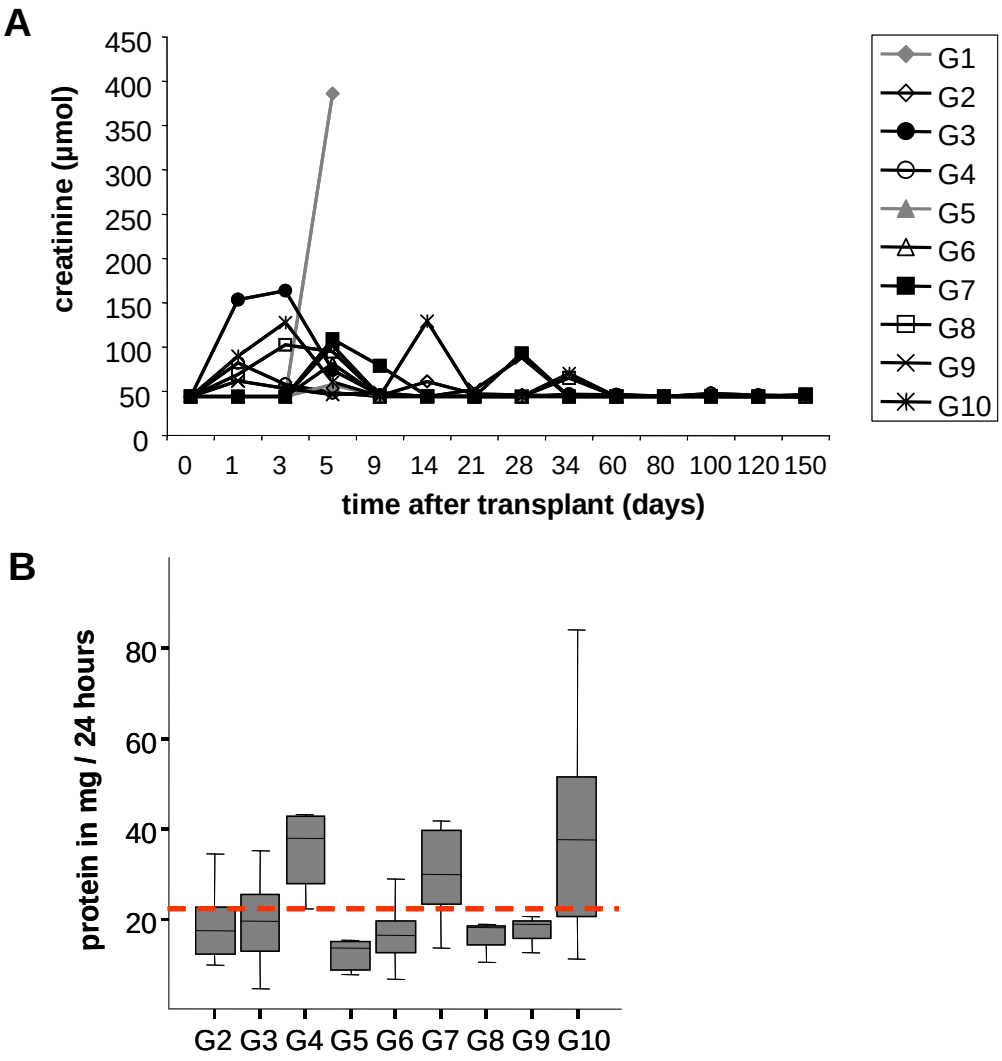


Figure 11. Serum creatinine and protein levels of recipients from experimental sub-groups

DA donor kidneys were transplanted into LEW recipients. The serum creatinine levels were determined at different time points, day 0, 1, 3, 5, 9, 14, 34, 60, 80, 100, 120, 130, 140, 150. **A) Serum creatinine levels. B) Protein levels on day 150 post-transplantation.** Unlike other groups, urine protein levels in the groups of prolonged cold ischemia time (G4), Maastricht strain RCMV infected at high dose (G7) and twice RCMV infection (G10) increased apparently at day 150 post-transplantation.

Graft survival: All recipients that received anti-CD4 mAb (G2) survived the 150-day observation period (**Figure 12**). However, in comparison with transplant recipients treated with anti-CD4 mAb (G2), further administration of R-CMV at a high viral dose (G7) significantly shortened survival time (p=0.0465), leading to a significant decrease of allograft survival rate. Furthermore, repeated R-CMV infection on day –9 and day 0 (G10) caused also a significant decrease of allograft survival (p=0.0149). By contrast, regardless of whether using Maastricht (G6) or England (G5) strain of R-CMV, infection at a low viral dose did not affect allograft survival.

To investigate whether the alteration of surgical conditions influence graft outcome, contralateral nephrectomy was performed on either day 0 or day 3 (G7 / G8,) and cold ischemia time was prolonged to mean time of 7 hours (G4 / G9). Nonetheless, unlike in the case of anti-CD4 mAb treated recipients (G2), prolonged cold ischemia time (G9) was unable to further influence graft survival (p=0.1298). These factors had no evident impact on recipients' graft survival (**Figure 12**).

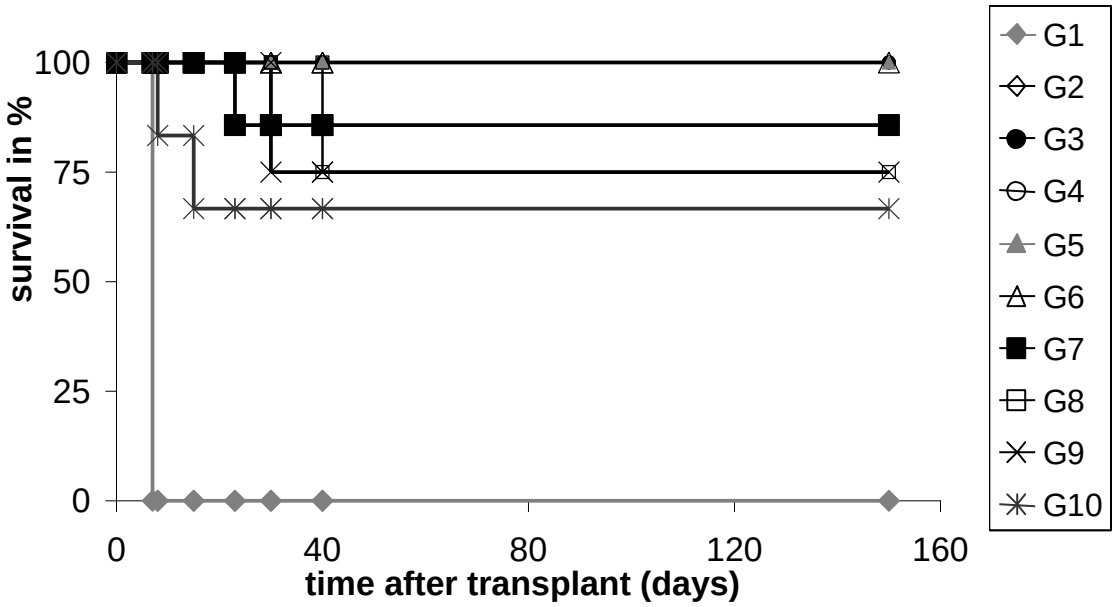


Figure 12. Effect of RCMV infection on allograft survival

The various groups (G1-G10) of recipients were treated with different regimens, as described in Materials and Methods. Allograft recipients that received anti-CD4 mAb (G2) permanently accepted their kidney grafts. Additional administration of R-CMV at a high viral dose (G7) or twice R-CMV infection at day –9 and day 0 (G10) caused significant decrease of allograft survival.

4.2.3. Histopathology

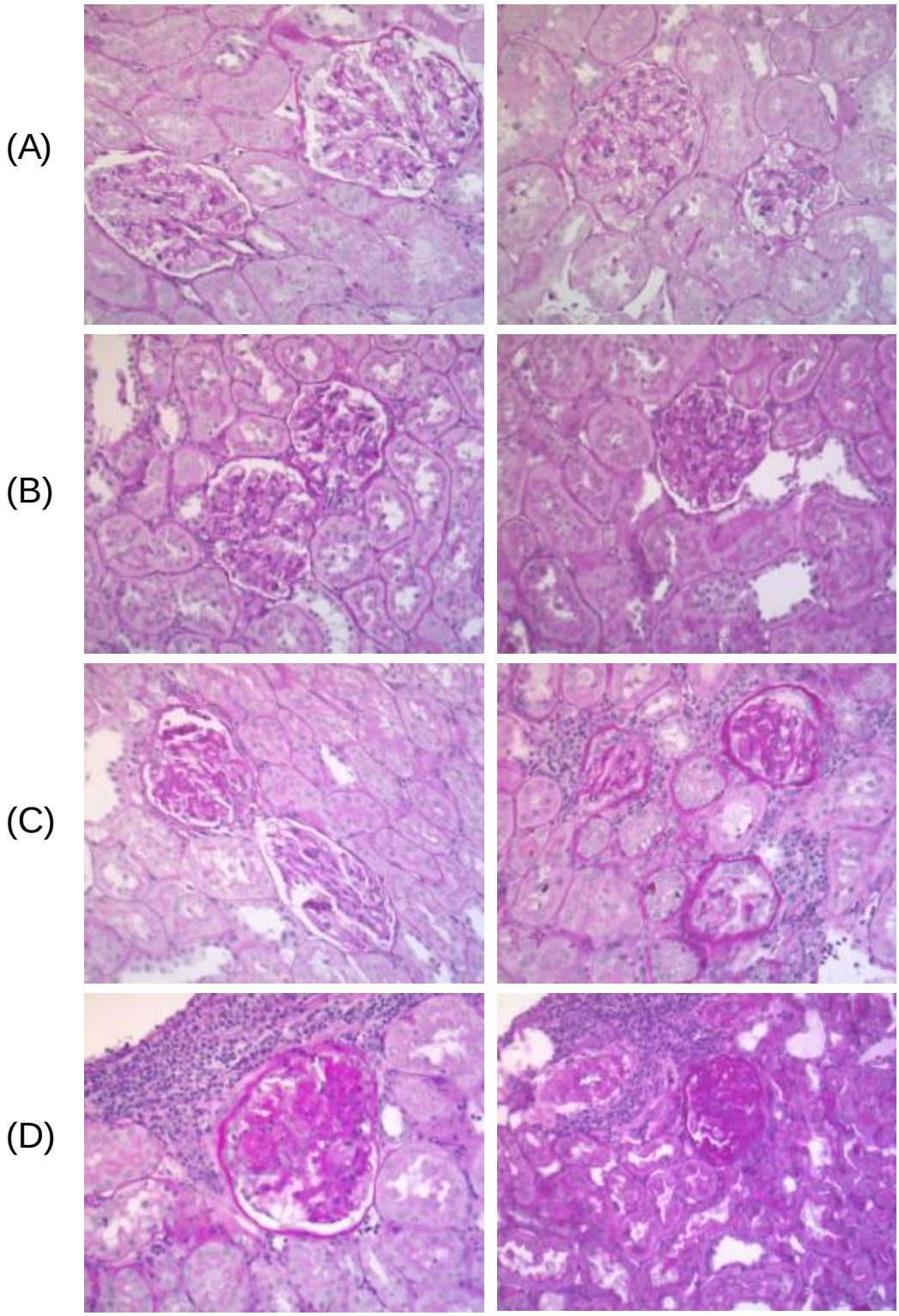


Figure 13. Graft histology

Glomerulosclerosis on day 150 post-transplantation in kidney allografts from groups G2 to G10 were assessed under microscope. A spectrum of specimen sections for different parts of allografts were analyzed on the basis of the following scale: **(A)** 0-25% glomerulus was sclerosed = grade 1. **(B)** 25-50% glomerulus was sclerosed = grade 2. **(C)** 50-75%

glomerulus was sclerosed = grade 3. **(D)** 75-100% glomerulus was sclerosed = grade 4. (PAS staining, X400)

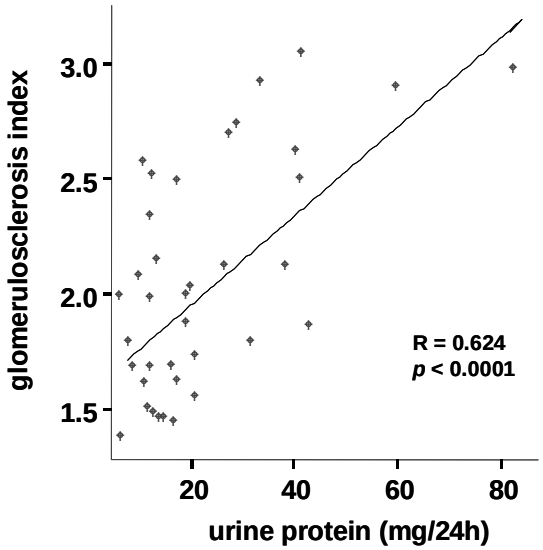


Figure 14. The association between proteinuria and glomerulosclerosis index of corresponding grafts

Urine protein levels were measured on day 150 post-Tx in kidney allografts from groups G2 to G10. Their corresponding glomerulosclerosis index was estimated. The alteration of urine protein levels correlates positively with the degree of glomerulosclerosis (Pearson correlation 0.624, p<0.0001).

Glomerulosclerosis is one prominent alteration found in chronic allograft nephropathy, characterized by increased extracellular matrix formation and cell proliferation. The process is irreversible and involves severe destruction of functioning nephrons (**Figure 13**) (101). The degree of glomerulosclerosis in different recipients from all the groups varied widely. To evaluate glomerulosclerosis index thoroughly, all long-term surviving grafts were evaluated in the present study, including all parts of each graft. The findings revealed a significant correlation between glomerulosclerosis index and urine protein level at day 150 (Pearson correlation 0.624, p<0.0001) (**Figure 14**), in agreement with previous research findings (102). Therefore, the level of 24-h urine protein may sensitively indicate the degree of glomerulosclerosis.

4.2.4. Intragraft gene expression

Previous studies described a few potential biomarkers associated with allograft rejection and tolerance induction. After kidney transplantation, a significant infiltration of intragraft CD25+ cells was observed in using biopsies (103). Rejection episodes closely correlated with increased transcript levels of Perforin and CTLA-4 (97) and the percentage of CD25+ and Foxp3+ cells (104) within kidney grafts. In addition, it was shown that high expression of CTLA-4 on the surface of CD4+ T cells in the peripheral blood indicates stable graft function in patients (105).

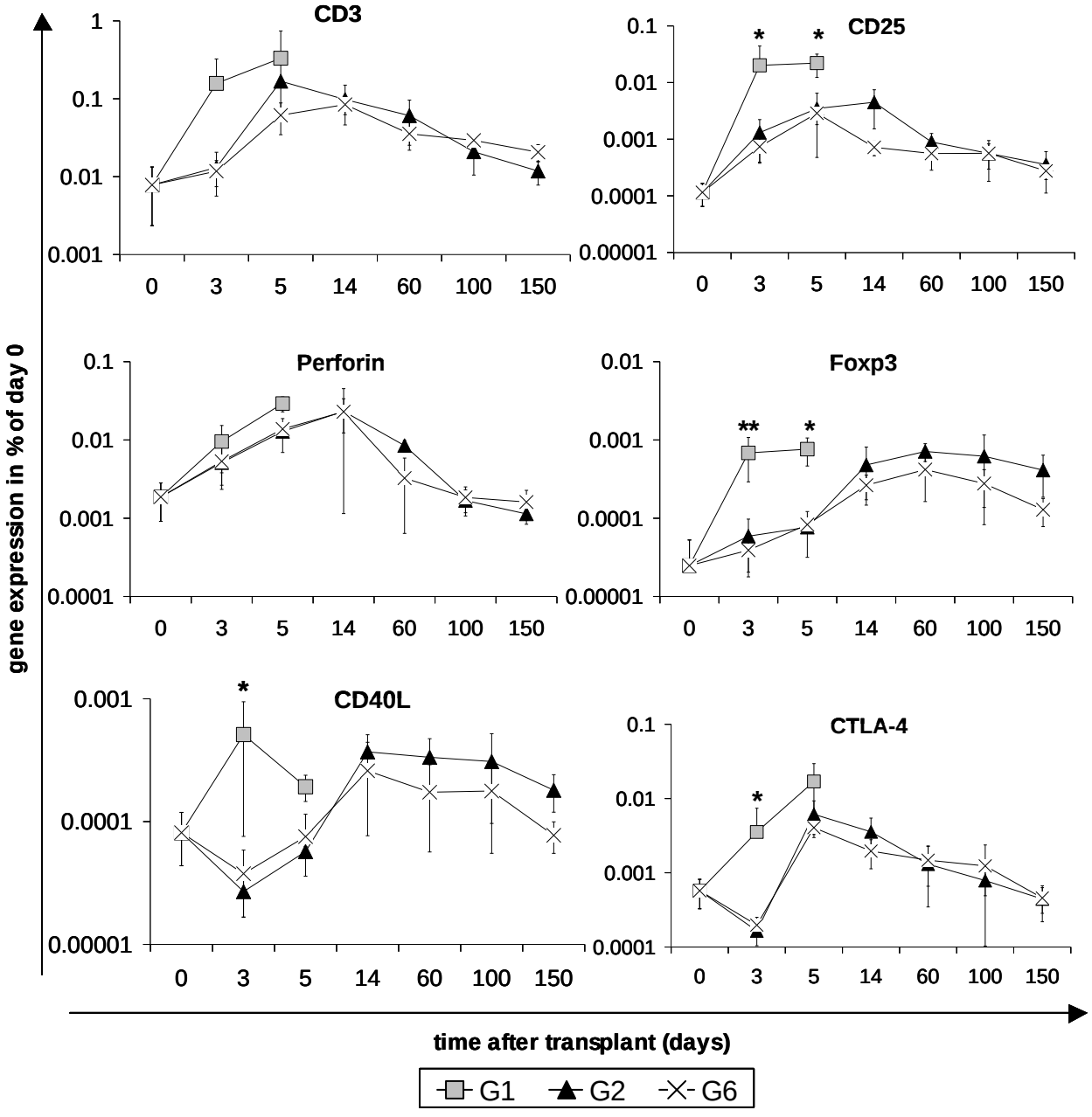


Figure 15. Intragraft CD3, CD25, Perforin, Foxp3, CD40L and CTLA-4 mRNA expression

after rat kidney transplantation

Allogeneic kidney transplantation of DA (RT1a) donor kidneys into LEW (RT1*l*) recipients was performed as described in Materials and Methods. CD3, CD25, Perforin, Foxp3, CD40L and CTLA-4 gene expression in grafts of untreated allograft (G1), anti-CD4 mAb-treated allograft recipients (G2), anti-CD4 mAb-treated and with a low dose of Maastricht strain R-CMV infected recipients (G6) was compared. Grafts were harvested for gene expression analysis on days 3, 5, 14, 60, 100 and 150 after transplantation. Naïve DA kidneys were harvested as controls to calculate the baseline expression levels (day 0). To analyze gene expression qRT-PCR was performed. Data are shown as mean ± SD.

Therefore, kinetic analysis of gene expression of these potential markers was serially tested in our model. As shown in figure 15, our results revealed that acute rejection in the untreated recipients induced a dramatic increase of intragraft CD3, CD25 and Perforin expression on days 3 and 5 after Tx (CD3, G1 day 0 vs. day 3: p=0.0635; G1 day 0 vs. day 5: p=0.0159; CD25, G1 day 0 vs. day 3: p=0.0159; G1 day 0 vs. day 5: p=0.011; Perforin, G1 day 0 vs. day 3: p=0.0317; G1 day 0 vs. day 5: p=0.0008). Initially, CD40L was also expressed highly but decreased between day 3 and day 5 (G1 day 0 vs. day 3: p=0.12; G 1 day 0 vs. day 5: p=0.01). Noticeably, to counterbalance the inflammatory responses Foxp3 and CTLA-4 expression was also upregulated constantly. In grafts transplanted into anti-CD4 mAb treated recipients, the increase in expression of CD3 and Perforin was inhibited, but their expression levels could not be differentiated from those in grafts transplanted into untreated recipients (CD3, G1 vs. G2 day 3: p=0.162; day 5: p=0.482; Perforin, G1 vs. G2 day 3: p=0.174; G1 day 5 vs. G2 day 14: p=0.653). The expression of CD40L and CTLA-4 was downregulated and significantly lower or marginally significantly than that of rejected grafts (day 3: CD40L, p=0.0159; CTLA-4, p=0.0952, respectively), and then concurrently increased to a high level on day 14. These increased levels were indistinguishable from levels of grafts from untreated acutely rejecting recipients (CD40L, G1 day 3 vs. G2 day 14: p=0.614; CTLA-4, G1 day 3 vs. G2 day 14: p=0.995; G1 day 5 vs. G2 day 14: p=0.119). By contrast to the rejected grafts, the increase in magnitude of CD25 and Foxp3 expression was dramatically dampened in grafts transplanted into anti-CD4 mAb treated recipients particularly at the early time point post-Tx (G1 vs. G2 day 3: CD25, p=0.0556; Foxp3, p=0.0079) (G1 vs. G2 day 5: CD25, p=0.0317; Foxp3, p=0.0159). Importantly, expression level of CD25 in

tolerated grafts during the whole observation period was always significantly lower than that of rejected grafts on day 5 post-Tx (G1 day 5 vs. G2 day 3, 5, 14, 60, 100, 150, p=0.0148, 0.0157, 0.0356, 0.0131, 0.011, 0.0114, respectively), whereas the level of Foxp3 gene expression of rejected recipients at day 3 or day 5 cannot be discriminated from that of tolerated recipients during day 14 to day 150 (G1 day 3 vs. G2 day 14, 60, 100, 150, p=0.553, 0.845, 0.758, 0.412, respectively)(G2 day 5 vs. G2 day 14, 60, 100, 150, p=0.326, 0.771, 0.698, 0.199, respectively). With respect to grafts transplanted into anti-CD4 mAb treated and RCMV infected recipients, Maastricht strain RCMV at a low dose did not induce evident alteration of gene expression patterns.

Previous studies also reported that gene expression of the receptor for hyaluronan-mediated motility (RHAMM) was correlated with chronic rejection in human renal allografts (106). Intragraft downregulation of Toag-1 may result from ongoing acute rejection episodes in rodent heart and kidney transplant models, which suggests that this is a sensitive predictor of rejection event (20). Apart from cytotoxic T lymphocytes, B lymphocytes and their specific antibodies also contribute to the formation of an allogeneic immune response. Anti-donor antibodies initiate acute humoral rejection but also contribute to some forms of chronic rejection (107). Intrarenal gene expression of CXCL13 (B lymphocyte chemokine) was massively upregulated in transplants with B-cell clusters, which is associated with acute interstitial rejection (108). Additionally, work performed by Siepert *et al.* first described downregulation of PDZK1 expression as being closely related with an acute rejection episode (Siepert *et al.*, unpublished observation). PDZK1 is expressed by parenchymal cells of the kidney (109).

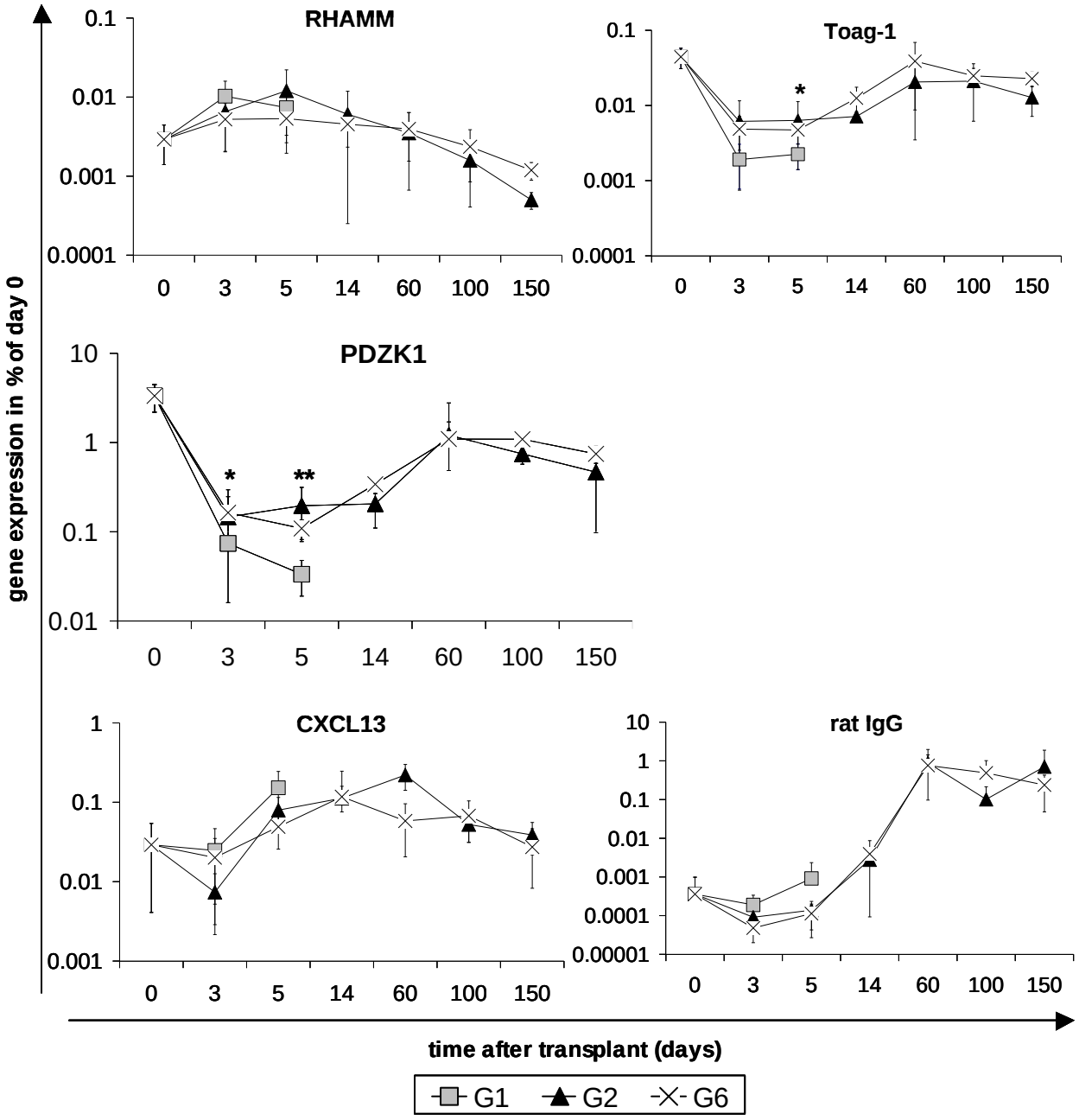


Figure 16. Intragraft RHAMM, Toag-1, PDZK-1, CXCL13 and IgG mRNA expression after rat kidney transplantation

Allogeneic kidney transplantation of DA (RT1a) donor kidneys into LEW (RT1*l*) recipients was performed as described in Materials and Methods. RHAMM, Toag-1, PDZK-1, CXCL13 and IgG gene fragment expression in grafts of untreated allograft (G1), anti-CD4 mAb-treated allograft recipients (G2), anti-CD4 mAb-treated and with a low dose of Maastricht strain R-CMV infected recipients (G6) was compared. Grafts were harvested for gene fragment expression analysis on days 3, 5, 14, 60, 100 and 150 after transplantation. Naïve DA kidneys were harvested as controls to calculate the baseline expression levels (day 0).

Data are shown as mean ± SD. Statistical significance is denoted by asterisks.

Our model was used to analyze whether the above-described gene expression could be utilized to monitor tolerance induction. Our findings revealed that expression of RHAMM, CXCL13 and rat IgG was upregulated in grafts transplanted into untreated recipients, while mRNA expression of PDZK-1 and Toag-1 was dramatically downregulated (RHAMM, G1 day 0 vs. day 3: p=0.0635; G1 day 0 vs. day 5: p=0.0977; CXCL13, G1 day 0 vs. day 3: p=0.905; G1 day 0 vs. day 5: p=0.0545; IgG, G1 day 0 vs. day 3: p=0.672; G1 day 0 vs. day 5: p=0.514)(PDZK-1, G1 day 0 vs. day 3: p=0.0156; G1 day 0 vs. day 5: p=0.0152; Toag-1, G1 day 0 vs. day 3: p=0.0117; G1 day 0 vs. day 5: p=0.0121) (**Figure 16**). Interestingly, similar expression pattern and level of RHAMM and CXCL13 during the first 5 days post Tx was observed in grafts transplanted into anti-CD4 treated recipients left uninfected or infected with low-dose CMV. Although rat IgG transcript level was transiently downregulated, it dramatically increased up to 300-fold on day 150 after Tx, which was significantly higher than the maximum level from grafts of untreated recipients (G2 day 150 vs. G1 day 5: p=0.0079; G6 day 150 vs. G1 day 5: p=0.0079). Interestingly, although mRNA expression of PDZK-1 and Toag-1 was transiently downregulated at the early stage in grafts of both anti-CD4 mAb treated groups (± low-dose CMV infection), their expression remained significantly higher compared to those of grafts transplanted into untreated recipients (day 3: Toag-1, G1 vs. G2: p=0.0952; G1 vs. G6: p=0.0771; PDZK-1, G1 vs. G2: p=0.6905; G1 vs. G6: p=0.0556) (day 5: Toag-1, G1 vs. G2: p=0.0268; G1 vs. G6: p=0.0761; PDZK-1, G1 vs. G2: p=0.0159; G1 vs. G6: p=0.01).

Thus, low dose CMV infection of anti-CD4 mAb treated recipients did not alter intragraft transcription of the analyzed genes. Furthermore, grafts of untreated acutely rejecting recipients are characterized by significantly lower intragraft Toag-1 and PDZK1 expression on day 5 after Tx.

4.2.5. Gene expression in peripheral blood

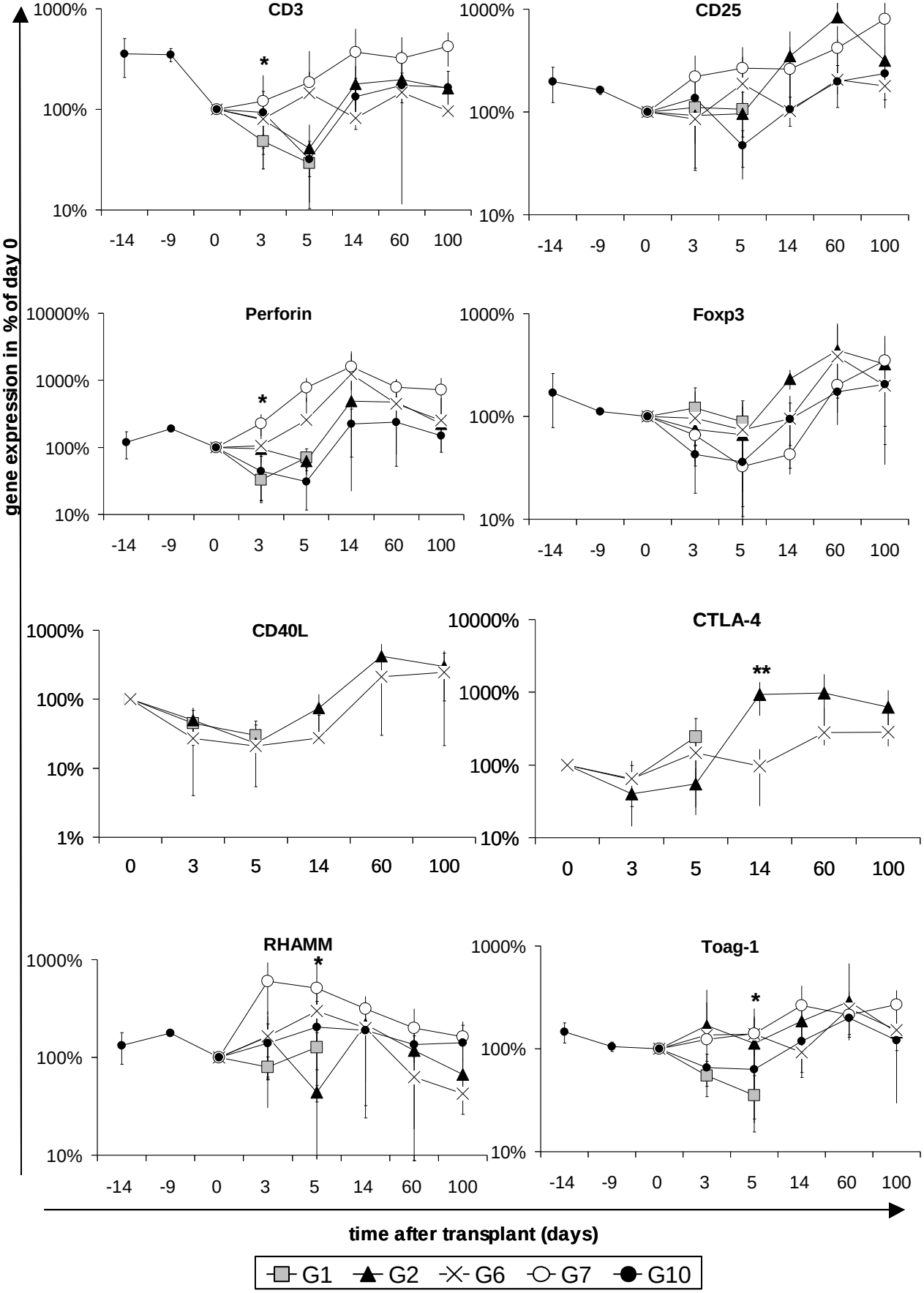


Figure 17. Gene expression in peripheral blood leukocytes of rat kidney graft recipients

Allogeneic kidney transplantation of DA (RT1a) donor kidneys into LEW (RT1*l*) recipients was performed as described in Materials and Methods. Recipients were sacrificed and peripheral blood was collected for gene expression analysis on days 0, 3, 5, 14, 60, 100, 150 after transplantation. To analyze mRNA expression, qRT-PCR was performed. CD3, CD25, Perforin, Foxp3, CD40L, CTLA-4, RHAMM and Toag-1 mRNA expression in the peripheral blood of the following groups was compared: untreated allogeneic kidney recipients (G1), anti-CD4 mAb-treated allogeneic kidney recipients (G2), anti-CD4 mAb-treated allogeneic kidney recipients infected with low dose Maastricht strain R-CMV (G6), anti-CD4 mAb-treated allogeneic kidney recipients infected with a high dose of Maastricht strain R-CMV (G7), anti-CD4 mAb-treated allogeneic kidney recipients infected twice with a high dose of Maastricht strain R-CMV (G10). Data are shown as mean ± SD.

Previously, a growing body of evidence suggested that monitoring the expression level of Perforin protein or mRNA in PBLs could be used to discriminate tolerant and rejecting kidney transplant patients with high specificity (90, 96, 110). Moreover, the presence of a significantly increased absolute number of $CD4^{+}CD25^{high}$ was detected in PBLs of rejection-free recipients compared with recipients with chronic graft dysfunction (111). Conversely, lower numbers of peripheral $CD4^{+}CD25^{high}Foxp3^{+}$ T cells was observed in patients with chronic rejection (112). In addition, the gene expression level of CD40L in PBLs was significantly upregulated in chronic allograft nephropathy (CAN) and acute rejection (113), whereas significantly lower CD40L expression and significantly higher intracellular CTLA-4 expression were detectable in patients with stable graft function (105), suggesting these molecules as noninvasive diagnostic biomarkers to monitor allograft function.

Therefore, in our model we further tested whether these transcripts together with RHAMM and Toag-1 mRNA in PBLs could be used as potential biomarkers for monitoring kidney graft function. Our results indicate that a considerable number of T lymphocytes infiltrated into kidney grafts of untreated recipients (G1), leading to an apparent reduction of mRNA expression of CD3, Perforin and CD40L in PBLs (CD3, day 0 vs. day 3: p=0.0001; day 0 vs. day 5: p=0.0017; Perforin, day 0 vs. day 3: p=0.0059; day 0 vs. day 5: p=0.0625; CD40L, day 0 vs. day 3: p=0.01; day 0 vs. day

5: p=0.0003) (**Figure 17**). In contrast, CD25 and Foxp3 expression maintained a relatively stable level in the peripheral blood early after transplantation (CD25, day 0 vs. day 3: p=0.518; day 0 vs. day 5: p=0.819; Foxp3, day 0 vs. day 3: p=0.577; day 0 vs. day 5: p=0.689). Peripheral RHAMM expression was unaffected in untreated recipients (day 0 vs. day 3: p=0.128; day 0 vs. day 5: p=0.358). Importantly, Toag-1 gene expression was remarkably downregulated in PBLs of untreated recipients (day 0 vs. day 3: p=0.0006; day 0 vs. day 5: p=0.00002), in line with reduced intragraft Toag-1 expression. In contrast, anti-CD4 mAb treatment delayed the transfer of T lymphocytes from peripheral blood into kidney graft, as expression levels of CD3 and Perforin on day 3 were marginally significantly or significantly higher than those of untreated recipients (G1 vs. G2 on day 3: CD3: p=0.0303; Perforin: p=0.0635), whereas no difference was observed on day 5 between samples of both groups (G1 vs. G2 on day 5: CD3: p=0.4286; Perforin: p=0.6905). Gene expression level of CD25, Foxp3, and CD40L in samples of anti-CD4 mAb treated recipients was similar to that of untreated recipient early after transplantation (day 3 and day 5) (G1 vs. G2 on day 3: CD25, p=0.511; Foxp3, p=0.277; CD40L, p=0.783; G1 vs. G2 on day 5: CD25, p=0.841; Foxp3, p=0.792; CD40L, p=0.514). Compared to samples of untreated recipients, peripheral RHAMM and Toag-1 expression level of anti-CD4 treated recipients was significantly reduced and increased accordingly on day 5 (G1 vs. G2 on day 3: RHAMM, p=0.5476; Toag-1, p=0.6991; day 5: RHAMM, p=0.0317; Toag-1, p=0.0519). Thus, peripheral gene expression level of CD3 and Perforin on day 3, RHAMM and Toag-1 on day 5 post-Tx may be potential biomarkers for monitoring allospecific immune responses. To further validate their diagnostic power, we analyzed their expression in our models of RCMV infection. Single infection with R-CMV at low or high dose (G6 and G7) uniformly induced a heightened expression of Perforin, whereas repeated R-CMV infection (G10) resulted in a transient downregulation of Perforin expression on day 3 or day 5 (day 3: G6 vs. G2, p=0.813; G7 vs. G2, p=0.0173; G10 vs. G2, p=0.147; day 5: G6 vs. G2, p=0.257; G7 vs. G2, p=0.0043; G10 vs. G2, p=0.0289). Thus Perforin transcription increases shortly after RCMV infection (G6, G7), whereas the mRNA expression decreases later after RCMV infection (G10) and in untreated recipients (G1). Similar expression pattern was observed for CD3 on day 5 (day 5: G6 vs. G2, p=0.6282; G7 vs. G2, p=0.0004; G10 vs. G2, p=0.5138). With respect to CD3 mRNA expression on day 3, the level of

R-CMV infection groups (G6, G7 and G10) was indistinguishable from that of anti-CD4 mAb treated recipients (day 3: G6 vs. G2, p=0.9452; G7 vs. G2, p=0.22; G10 vs. G2, p=0.931). Interestingly, RHAMM expression of recipients infected once with RCMV (G6, G7) increased after transplantation and was higher in comparison to that of untreated recipients on day 5. In addition, we detected an increase in CTLA-4 transcription in PBL samples of untreated recipients. This increase was delayed but reached higher levels in samples of anti-CD4 treated recipients. In contrast, RCMV infection prevented this increase in CTLA-4 transcription on day 14 (G6 vs. G2: p=0.0079). Noticeably and importantly, peripheral blood Toag-1 transcription in recipients after single infection with RCMV at different doses was significantly higher than that of untreated recipients at day 5 (G6 vs. G1: p=0.0140; G7 vs. G1: p=0.0022), while Toag-1 mRNA level of twice CMV-infected recipients (=high incidence of acute and chronic rejection) was downregulated on day 5 and could not be distinguished from that of rejected recipients (G10 vs. G1: p=0.2403) or tolerance developing recipients (G10 vs. G2: p=0.2571). These findings were in line with not only their long-term graft outcome but also with intragraft Toag-1 gene expression profiles.

Nevertheless, to better evaluate peripheral Toag-1 gene expression levels as an early biomarker of acute and chronic rejection, all transplanted recipients were regrouped into either acute rejection (AR = recipients died within 40 days after Tx), chronic rejection (CR = recipients were characterised by proteinuria $\geq$ 20mg on day 150 post-Tx) or tolerance induction (TI = recipients survived observation period and had no proteinuria). Toag-1 expression levels were reanalyzed correspondingly (**Figure 18, A**). Although Toag-1 expression was already reduced on day 3 after Tx in PBLs of acutely rejecting recipients compared to samples of CR and TI recipients, this was not statistically significant (AR versus CR: p=0.4942, AR versus TI: p=0.0845, TI versus CR: p=0.4002). However, Toag-1 transcript level in samples of the TI group on day 5 was significantly higher compared to that in samples of the AR group (p=0.0067) or the CR group (p=0.0089). Interestingly, no difference of Toag-1 gene expression between AR and CR groups was observed (p=0.3418). Furthermore, the gene expression level of Toag-1 at day 5 correlated with urine protein level at day 150 post-Tx (p=0.021) or glomerulosclerosis of transplanted allografts (p<0.0001)

(**Figure 18, B and C**). Moreover, intragraft Toag-1 gene expression level in TI group at day 150 was significantly higher in comparison with the CR group (data not shown). Therefore, the level of Toag-1 gene expression on day 5 post-Tx can predict long-term graft outcome.

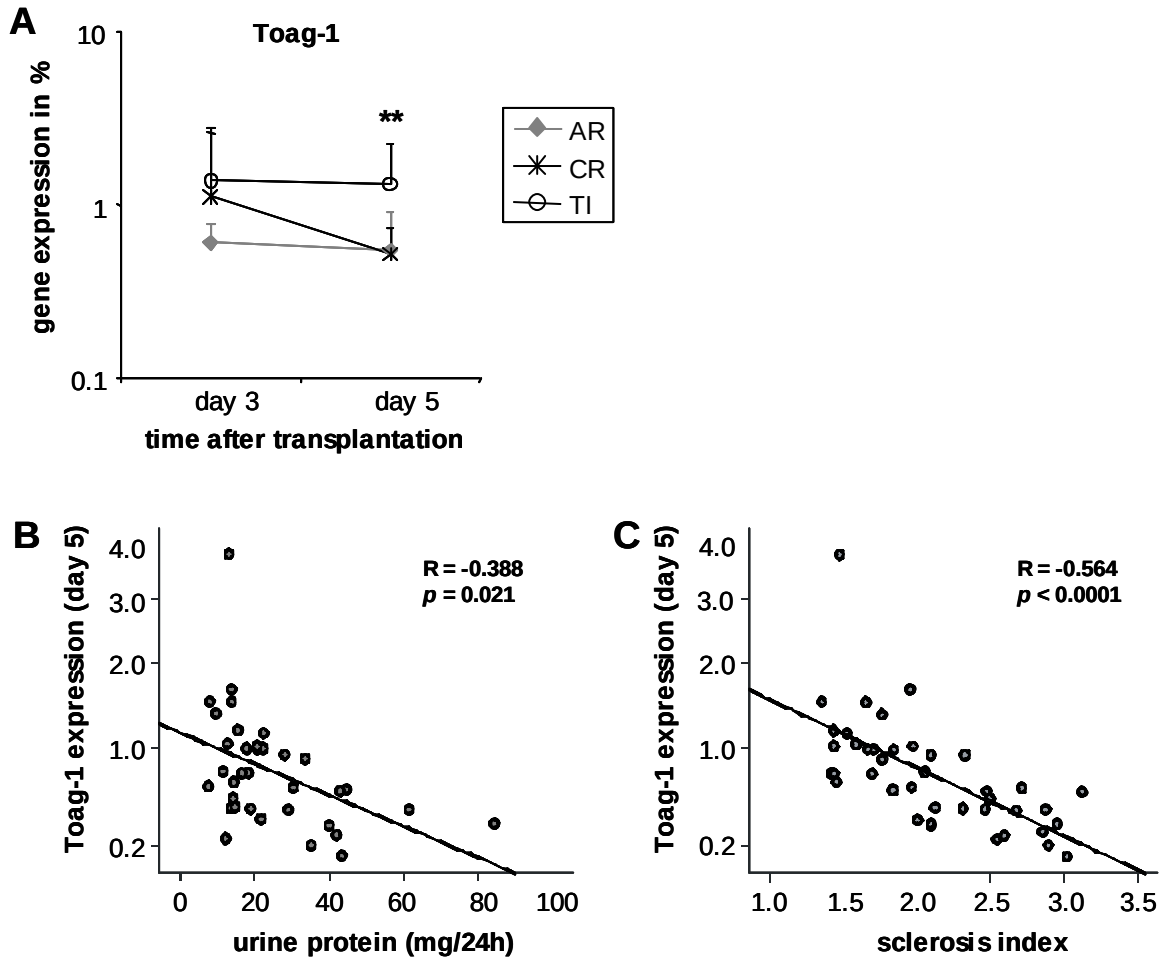


Figure 18. Early gene expression of peripheral Toag-1 in the groups of acute rejection (AR), chronic rejection (CR) and tolerance induction (TI) and correlation between Toag-1 gene expression level at day 5 post-Tx and glomerulosclerosis index of corresponding grafts or urine protein level

Gene expression of Toag-1 in the peripheral blood was analyzed by RT-PCR at day 3 and day 5 post-Tx from groups G2 to G10. Their corresponding glomerulosclerosis index and urine protein levels were assessed at day 150 post-Tx in kidney allografts. (A) all transplanted recipients were regrouped into AR, CR or TI. Peripheral Toag-1 mRNA level in samples of the TI group on day 5 was significantly higher than that in samples of the AR group (p=0.0067) or the CR group (p=0.0089). The alteration of Toag-1 gene expression levels correlates negatively with (C) the degree of glomerulosclerosis (Pearson correlation –

0.564, p<0.0001) and (B) the levels of urine protein (Pearson correlation –0.388, p=0.021).

4.2.6. Allo-antibody production

Previous studies revealed that development of donor-specific allo-antibodies could influence graft outcome (114). To test whether development of allo-antibodies is associated with ongoing allograft rejection, kinetic monitoring of circulating allo-antibodies was performed in our experimental groups.

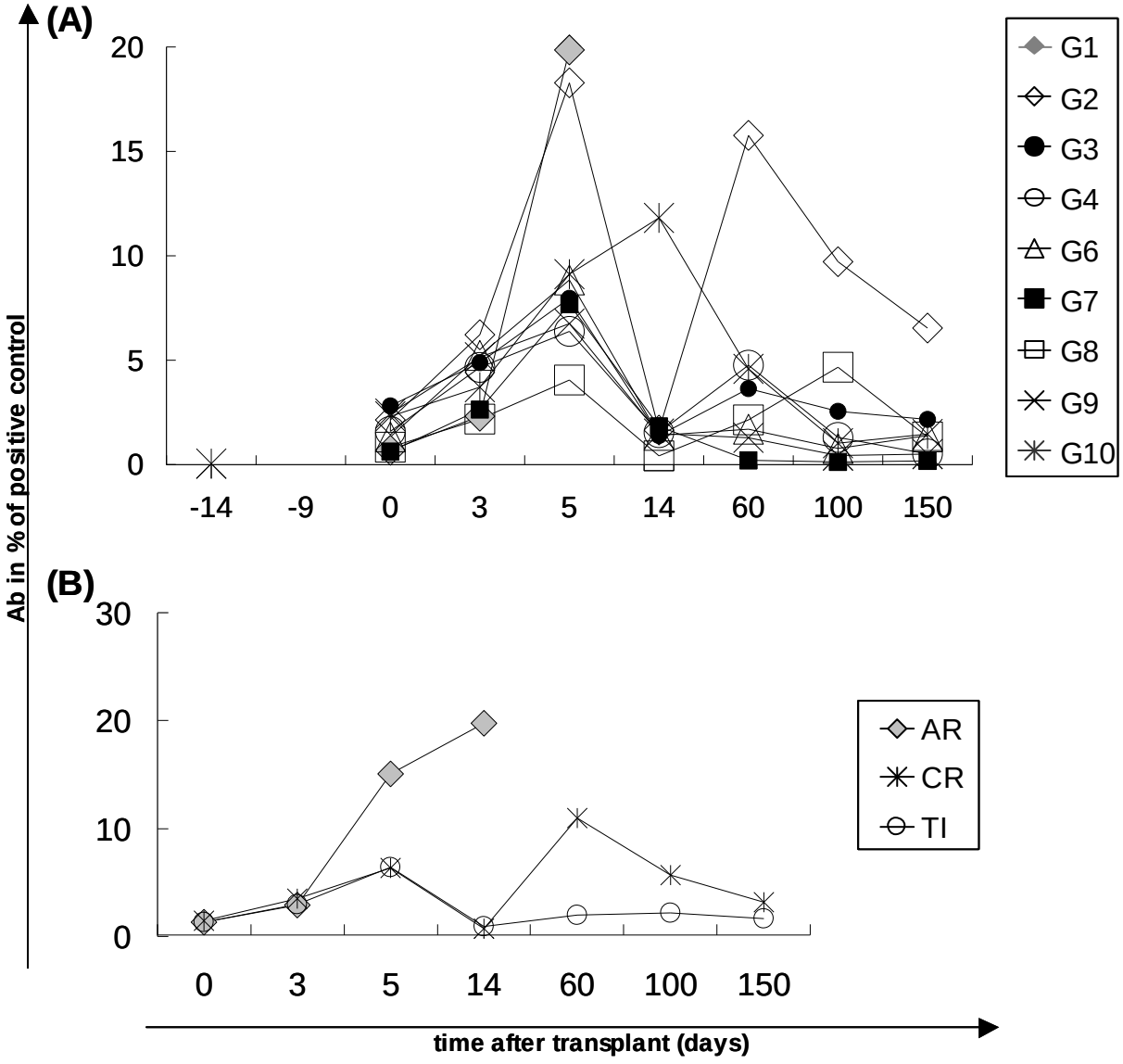


Figure 19. A flow cytometric analysis of circulating donor-specific allo-antibodies

After transplantation, peripheral blood sera were serially collected at days 0, 3, 5, 14, 60, 100, 150 (including day –14 and day –9 for CMV twice infection group). Circulating donor-specific

allo-antibodies in 1/10 dilutions of sera were analyzed by flow cytometry method and reported as % alloantibody production in relation to a positive control (serum of a Lewis rat immunized with DA splenocytes, see also Materials and Methods).

Our findings revealed that an increase in production of IgG allo-antibodies did indeed occur in the untreated recipients (G1) particularly on day 5 (**Figure 19 A**, day 0 vs. day 5: 0.687±0.458 vs. 19.858±5.359, p=0.0022), but could not be differentiated from that of anti-CD4 mAb treated recipients on day 5 (p=0.6282). This indicates formation of peripheral allo-antibodies at an early stage after transplantation. Whether the allo-antibodies contribute to the acute rejection of the allogeneic kidney grafts needs to be further investigated. Interestingly, additional RCMV infection caused a marginally significantly or significantly lower production of allo-antibodies at day 5 (G6 vs. G2: p=0.0728; G7 vs. G2: p=0.0175; G8 vs. G2: p=0.0025; G9 vs. G2: p=0.0242; G10 vs. G2: p=0.035, respectively). Moreover, we detected a drastic increase in the levels of IgG production in one anti-CD4 mAb treated recipient which was infected twice with CMV (G10) on day 14 and in one anti-CD4 mAb only treated recipient (G2) on day 60 after Tx. Interestingly, these two recipients experienced an acute rejection and chronic rejection, respectively. Although the production of IgG allo-antibodies on day 3 or day 5 after Tx was maintained at a low level for that anti-CD4 mAb only treated recipient (G2), a conspicuous augmentation of serum IgG allo-antibody production was detected 90 days prior to the subsequent increase of serum creatinine, indicating the importance of longitudinal serum allo-antibody monitoring in predicting some forms of chronic rejection. To further evaluate the validity of the detection of circulating donor-specific allo-antibodies as a biomarker, all the transplanted recipients were regrouped into acute rejection group (AR), chronic rejection group (CR) and tolerance induction group (TI) according to the features described earlier. Compared to TI group, the level of serum IgG allo-antibodies in the CR group did not increase significantly during the entire experimental observation (CR vs. TI: day 3, 5, 14, 60, 100, 150, p=0.447, 0.954, 0.213, 0.277, 0.457, 0.423, respectively). Furthermore, although the level of serum IgG allo-antibodies on day 3 in the acute rejection group cannot be discriminated from that either in chronic rejection group (day 3: p=0.2875) or in tolerance induction group (day 3: p=0.7427), there were marginally significant differences detectable on day 5 (AR vs. CR:

p=0.0683; AR vs. TI: p=0.0660), suggesting that the increased level of serum IgG allo-antibodies on day 5 post-Tx is a risk factor and could be a potential biomarker predicting developing graft dysfunction.

4.2.7. Peripheral T cell apoptosis

During the induction of stable tolerance to MHC incompatible allografts, apoptosis is often required to reduce allospecific immune response, particularly apoptosis of effector T cells (115). Interestingly, over-expression of Toag-1 led to a higher susceptibility to apoptosis in murine $CD4^+$ T cells (Keeren *et al.* Journal of Immunology-in revision). As untreated acutely rejecting recipients and anti-CD4 mAb treated tolerance developing recipients are characterised by differences in peripheral Toag-1 transcription, they could also show differences in peripheral T cell apoptosis after transplantation. In order to address the question whether T cell activation in untreated acutely rejecting recipients is correlated with decreased percentage of peripheral T cells undergoing apoptosis, we studied the peripheral T cell composition and their susceptibility to spontaneous apoptosis 5 days after transplantation.

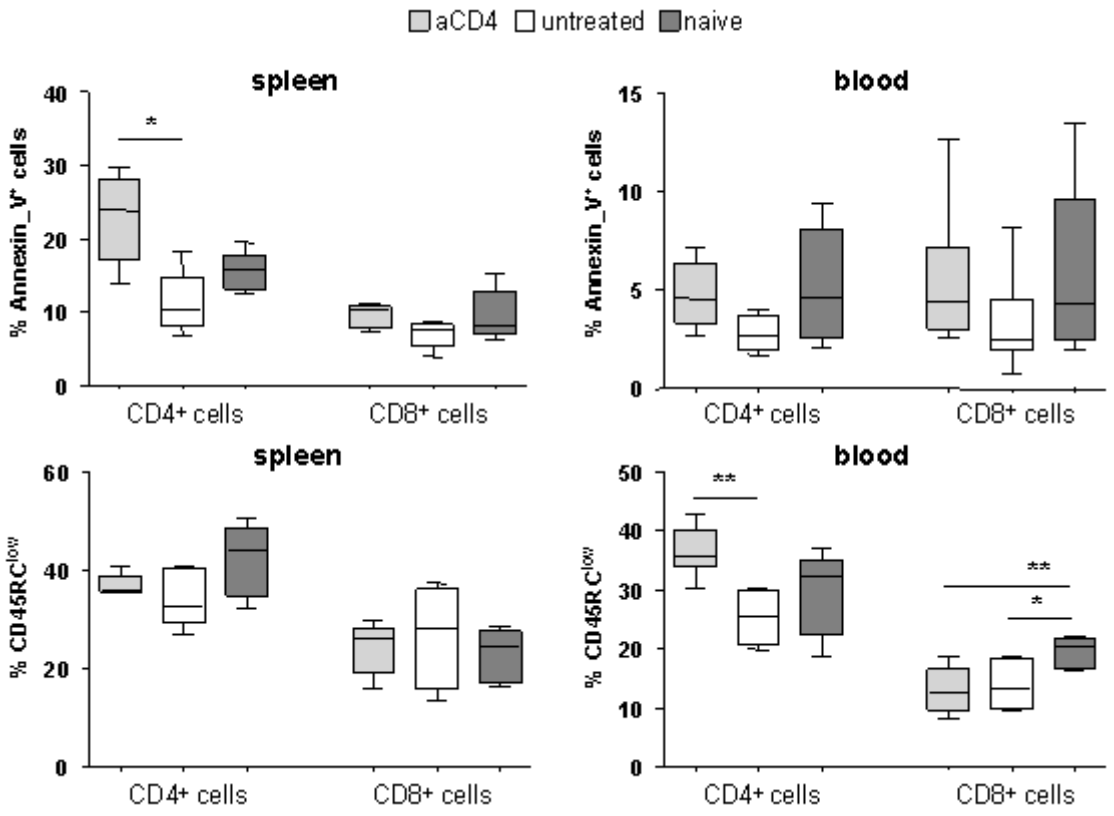


Figure 20. Decreased spontaneous apoptosis of $CD4^+$ T cells and frequency of

CD45RC^{low} cells in untreated acutely rejecting recipients

Frequency of Annexin_V positive T cells in splenocytes and peripheral blood of untreated (G1), anti-CD4 treated (G2) kidney graft recipients on day 5 post-Tx and naïve Lewis rats. Frequency of CD45RC^{low} T cells in splenocytes and peripheral blood of untreated (G1), anti-CD4 treated (G2) kidney graft recipients on day 5 post-Tx and naïve Lewis rats. Statistical significance is denoted by asterisks.

As shown in figure 20, the capability of the anti-CD4 mAb RIB5/2 to induce transplantation tolerance was characterized by an elevation of CD4^{+}Annexin_V^{+} T cells in the spleen but not in the periphery (untreated recipients vs. anti-CD4 mAb tolerated recipients: p=0.0267, naïve control vs. anti-CD4 mAb tolerated recipients: p=0.0391). However, the frequency of CD8^{+}Annexin_V^{+} T cells was not influenced. Conversely, a slight reduction of CD4^{+}Annexin_V^{+} T cells was detected in the spleen of untreated recipients as compared to samples of naïve animals although this was not statistically significant (p=0.498), implying that the alloreactive immune response inhibits the emergence of T cell apoptosis.

To analyse the differences in the distribution of specific T cell sub-populations involved in tolerance induction by RIB5/2 treatment, flow cytometric analysis in the periphery and in the spleen on day 5 after Tx was performed. We have compared the percentage of T cells expressing CD4 and low level of CD45RC in spleen and peripheral blood. The CD4^{+}CD45RC^{high} T-cell (Th1-like) subset is mainly responsible for the allo-specific immune response (116), while the CD4^{+}CD45RC^{low} T cell subset also contains regulatory T cells and does not function as pathogenic alloreactive T cells. The percentage of CD4^{+}CD45RC^{low} in the peripheral blood of anti-CD4 mAb treated recipients increased significantly compared to that in naïve animals (p=0.0341) and was dramatically higher compared to that in untreated recipients (p=0.000434). There was an apparent reduction of CD4^{+}CD45RC^{low} T cells detectable in untreated recipients compared to naïve animals although this was not statistically significant (p=0.0692) (**Figure 20**). Furthermore, we detected a relatively reduced frequency of CD8^{+}CD45RC^{low} T cells in the group of no treatment (p=0.0012) but also in the group of anti-CD4 mAb treatment (p=0.0054). No statistically significant differences for the frequencies of CD4^{+}CD45RC^{low} or CD8^{+}CD45RC^{low} T

cells were found in the spleen (**Figure 20**).

4.3. Tolerance abrogation induced by exogenous IL-2

4.3.1. Effect on graft function

All recipients of groups 15, 16 and 17 received anti-CD4 mAb treatment. It has been previously shown that ex vivo and in vivo IL-2 treatment can lead to impaired allograft function and survival (22). Within our study, the use of exogenous IL-2 led to an obvious urinary concentrating defect with marked polyuria. The urine volume of the recipients receiving two different doses of IL-2 was significantly higher compared to that of recipients receiving no IL-2 during the whole observation period (G15 vs. G 16 on day 0, p=0.866; day 10, p=0.21; day 20, p=0.0325; day 30, p=0.202; day 40, p=0.06; day 50, p=0.045)(G15 vs. G 17 on day 0, p=0.205; day 10, p=0.0159; day 20, p=0.0029; day 30, p=0.049; day 40, p=0.0329; day 50, p=0.0248). The increase of urine output was IL-2 dose dependent. A higher dose of IL-2 elicited a considerable higher volume of urine. However, administration of exogenous IL-2 did not cause a significant alteration of urine protein production (G15 vs. G 16 on day 0, p=0.327; day 10, p=0.853; day 20, p=0.896; day 30, p=0.3; day 40, p=0.83; day 50, p=0.942)(G15 vs. G 17 on day 0, p=0.114; day 10, p=0.232; day 20, p=0.917; day 30, p=0.598; day 40, p=0.862; day 50, p=0.591) (**Figure 21**).

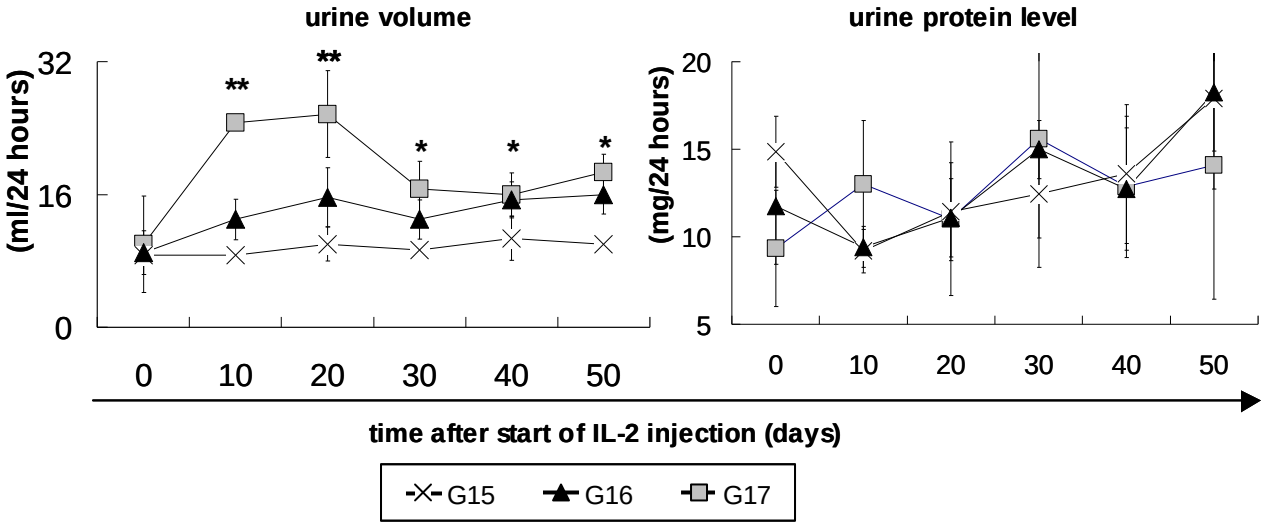


Figure 21. 24-hour urine volume and urine protein level

24-h urine outputs and urine protein levels of untreated (G15) and low dose IL-2-treated (G16) and high dose IL-2-treated recipients (G17) were determined at different time points, days 0, 1, 3, 5, 9, 14, 34, 60, 80, 100, 120, 130, 140 and 150 after start of exogenous IL-2 injection. Initial urine volume and urine protein level at day 0 were comparable in all groups. Exogenous IL-2 administration resulted in a significant increase of urine output ($p<0.05$) but no alteration of urine protein production ($p>0.05$).

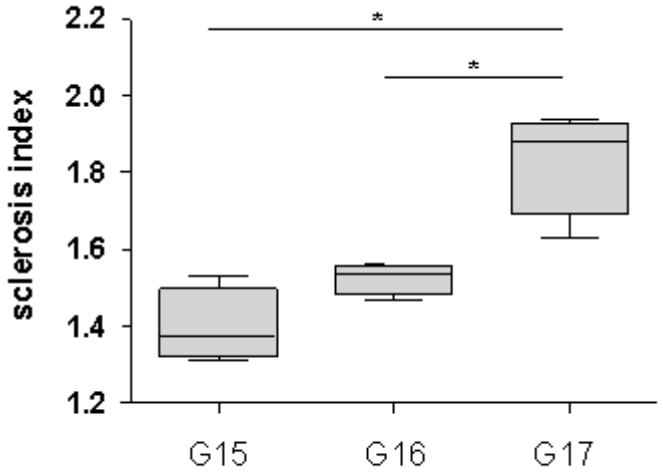


Figure 22. Comparative analysis of the degree of glomerulosclerosis in the grafts
Glomerulosclerosis on day 150 post-transplantation in kidney allografts from untreated recipients (G15), recipients receiving a low dose of IL-2 (G16), recipients receiving a high dose of IL-2 (G17) groups were assessed under microscope. The sclerosis index of untreated recipients is significantly lower than that of high IL-2 treated recipients ($p=0.0225$), similar to that of low IL-2 treated recipients ($p=0.169$).

As shown in figure 22, glomerusclerosis analysis of grafts revealed that sclerosis index in the group of high dose IL-2 treatment was significantly higher compared to that in the no treatment group ($p=0.0225$) or in the low dose IL-2 treatment group ($p=0.0413$). But no obvious difference was observed between untreated recipients and low dose IL-2 treated animals ($p=0.169$).

4.3.2. Gene expression in peripheral blood

Use of exogenous IL-2 did not induce any remarkable alteration of CD3 gene expression in the peripheral blood early after injection. However, we detected a significant down-regulation of CD3 transcription on day 10 post-injection (**Figure 23**,

day 10: no treatment vs. low IL-2 injection, p=0.038, no treatment vs. high IL-2 injection, p=0.0304). Interestingly, there was no significant difference between low- and high-IL-2 injection groups (p=0.6206).

Perforin is a key effector of T cell mediated cytotoxicity. Addition of IL-2 can directly regulate Perforin gene expression in $CD8^+$ T cells and primary NK cells (117, 118). In our study, Perforin transcript levels in the peripheral blood increased slightly after administration of a high dose of IL-2 to anti-CD4 mAb treated recipients. However, this increase was not statistically significant (G15 vs. G17 on day 2: p=0.263; day 5: p=0.128; day 10: p=0.432) (**Figure 23**).

The previous investigation (CMV infection) showed that enhanced alloreactive responses increased RHAMM expression (**Figure 16**). In the present study, high dose of exogenous IL-2 induced an increase of RHAMM expression on day 5 and day 10. But no statistical differences among the three groups studied could be observed during the whole observation period (day 2: p=0.297; day 5: 0.779; day 10: p=0.148).

The percentage of T lymphocytes expressing CD25 and Foxp3 was correlated with kidney graft function (104). A severe allospecific immune response could induce an increase of intragraft and peripheral CD25 and Foxp3 transcription (**Figure 15 and 17**). In particular, intragraft CD25 expression levels could discriminate tolerated grafts from rejected grafts (**Figure 15**). Our present study revealed that administration of exogenous IL-2 caused an increase in CD25 mRNA expression on day 2 and day 5 in a dose dependent manner although this was again not significant (G15, G16 and G17: day 2, p=0.831; day 5, p=0.803). This increase in CD25 transcription was followed by a significant decrease on day 10 (G15, G16 and G17: p=0.0665), which was interestlingly observed among these three groups, particularly between untreated group and high IL-2 injection group (G15 vs. G17, p=0.029). Exogenous IL-2 also induced a transient increase in Foxp3 expression (**Figure 23**). Interestingly this increase in Foxp3 transcription was not dependent on the IL-2 amount, used as both doses caused a similar 2-fold increase in Foxp3 transcription. However, these differences in Foxp3 mRNA expression did not reach statistical significance (Foxp3 on day 2: p=0.951; day 5: p=0.971; day 10: p=0.177).

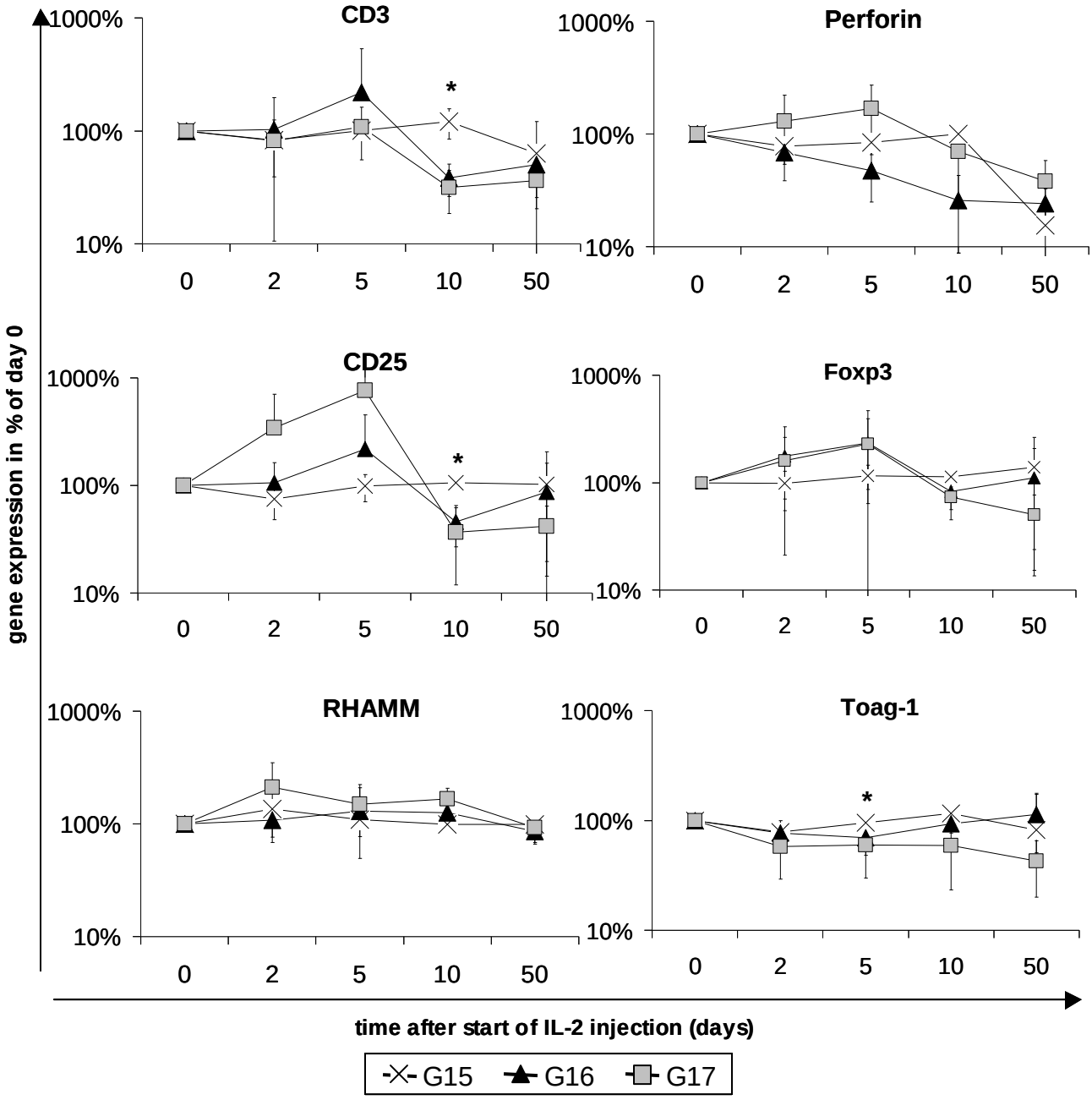


Figure 23: Gene expression analysis in the peripheral blood

Allogeneic kidney transplantation of DA ($RT1^a$) donor kidneys into anti-CD4 mAb treated LEW ($RT1^l$) recipients was performed as described in Materials and Methods. Exogenous IL-2 at high or low dose was applied continuously for 10 days to two different groups starting on day 100 post-Tx. The peripheral blood lymphocytes were isolated at days 0, 2, 5, 10, 50 after start of IL-2 injection. The gene expression of CD3, Foxp3, Perforin, RHAMM and Toag-1 in the peripheral blood was analyzed by RT-PCR and the data were compared between the untreated group (G15), low dose IL-2 group (G16) and high dose IL-2 group (G17).

Previous studies and the results presented earlier in the RCMV infection model indicated that Toag-1 is a useful marker to monitor allospecific immune responses and predict graft outcome (20) (**Figure 17**). Indeed, during the whole course of IL-2 administration, Toag-1 transcription was decreasing, particularly on day 5 and day 10 (**Figure 23**). Toag-1 transcript levels were significantly lower in samples of high dose IL-2 treated recipients (day 5, p=0.043; day 10, p=0.1) but also in samples of low dose IL-2 treated recipients (day 5, p=0.0396; day 10, p=0.241) compared to samples of recipients receiving no IL-2. Unfortunately, gene expression of Toag-1 in samples of high dose IL-2 treated recipients could not be discriminated from those of low dose IL-2 treated recipients (day 5, p=0.566; day 10, p=0.288) (**Figure 23**). A combinational analysis of Toag-1 and RHAMM transcription allowed an even better discrimination of untreated and high dose IL-2 treated recipients (G15 vs. G17: day 5, p=0.07; day 10, p=0.0289), whereas there was no difference of Toag-1/RHAMM transcript levels between no treatment group and low dose IL-2 treatment group (G15 vs. G16: day 5, p=0.878; day 10, p=0.162) (**Figure 24**). Therefore, analysis of Toag-1 transcription and the ratio of Toag-1 and RHAMM will be of great value when assessing allospecific immune responses and predicting graft function.

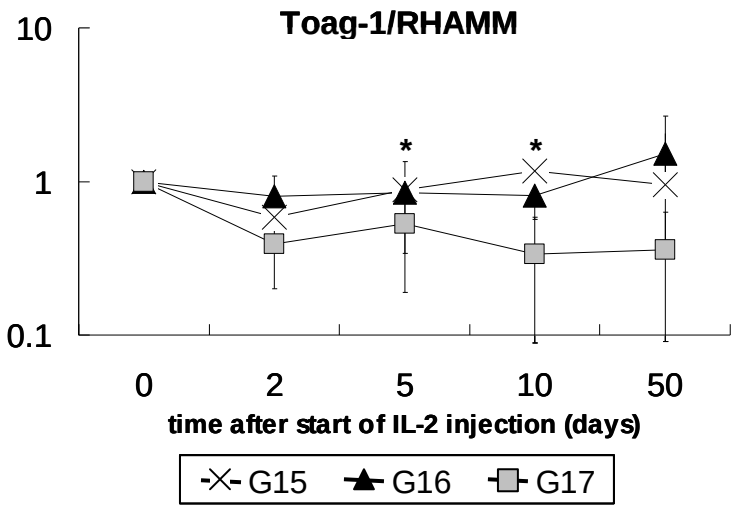


Figure 24. Combinational analysis of Toag-1 and RHAMM expression

The gene expression Toag-1 in the peripheral blood at days 0, 2, 5, 10, 50 after start of IL-2 injection was divided by that of RHAMM at their corresponding time points. The ratio of Toag-1/RHAMM gene expression of high IL-2 treated recipients is statistically lower than that of untreated recipients (p=0.0289).

4.3.3. FACS analysis of CD25+Foxp3- cells in blood

As we detected a transient increase in CD25 and Foxp3 transcription after IL-2 injection, we compared the relative frequencies of $CD4^+CD25^+Foxp3^-$ and $CD4^+CD25^+Foxp3^+$ T cells in PBLs of our recipients at the end of the observation.

Significant elevation of relative and absolute numbers of $CD4^+CD25^+Foxp3^-$ T cells in the peripheral blood was observed in the high dose IL-2 treatment group, compared to that in the no-treatment group or in the low dose IL-2 treatment group (no treatment vs. low IL-2: p=0.896, low IL-2 vs. high IL-2: p=0.0467, no treatment vs. high IL-2: p=0.01) (**Figure 25**). Conversely, there was no remarkable alteration of relative and absolute numbers of $CD4^+CD25^+Foxp3^+$ T cells in the peripheral blood (no treatment vs. low IL-2: p=0.514, low IL-2 vs. high IL-2: p=0.569, no treatment vs. high IL-2: p=0.771).

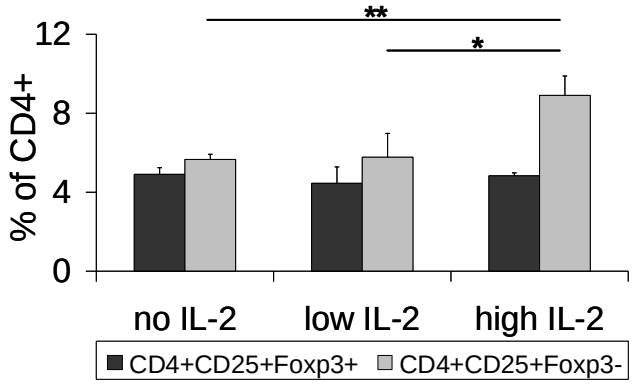


Figure 25. FACS analysis of peripheral blood lymphocytes

The peripheral blood was collected at the end of the observation period (50 days after the first IL-2 injection) for flow cytometric analysis. Significant elevation of relative and absolute numbers of $CD4^+CD25^+Foxp3^-$ T cells in the peripheral blood was observed in the high dose IL-2 treatment group, compared to that in the no-treatment group or in the low dose IL-2 treatment group (no treatment vs. low IL-2: p=0.896, low IL-2 vs. high IL-2: p=0.0467, no treatment vs. high IL-2: p=0.01). Statistical significance is denoted by asterisks. And there was no statistical difference of relative and absolute numbers of $CD4^+CD25^+Foxp3^+$ T cells in the peripheral blood (no treatment vs. low IL-2: p=0.514, low IL-2 vs. high IL-2: p=0.569, no treatment vs. high IL-2: p=0.771).

4.3.4. Immunohistochemistry

Monoclonal antibody (mAb) ED1, recognizing exclusively cells of the mononuclear phagocyte system, was used widely as a marker for rat monocytes/macrophage (119). The amount of ED1 expression usually correlates with phagocytic activity (119). The immunohistochemical analysis demonstrated that intragraft cellular infiltrates of ED1+monocytes/macrophages in the recipients receiving a high dose of IL-2 (**Figure 26, a**) were obviously more intensive than those in recipients receiving a low dose of IL-2 treatment (**Figure 26, b**). Thus, the presence of macrophages was highly correlated with ongoing chronic rejection of allografts.

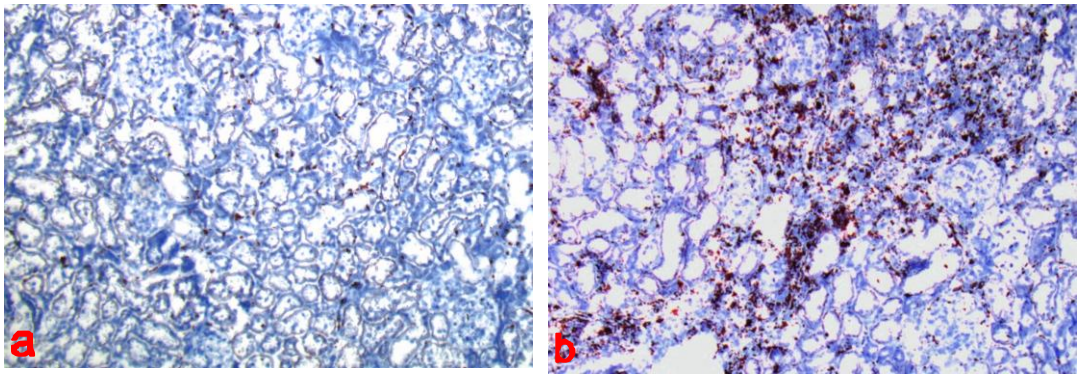


Figure 26. Immunohistochemical analysis of ED-1.

Allografts harvested at the end of the observation period (50 days after the first IL-2 injection) were snap frozen into Tissue Tec and stained for ED-1 protein as described in Materials and Methods. (x 400 of original magnification). $ED1^{+}$ cell accumulation in allografts treated with a high dose of IL-2 (a) was evidently more intensive than in the allografts treated with a low dose of IL-2 (b).

5. DISCUSSION

Although anti-CD4 mAb-induced permanent allograft acceptance, additional administration of exogenous IL-2, R-CMV infection at high viral dose (G7) and repeated R-CMV infection on day –9 and day 0 (G10) could lead to impaired graft function. Moreover, apart from external effect, intrinsic non-immunological factors such as weight difference between donor and recipient could also affect early kidney graft function. Therefore, monitoring of allograft acceptance and function is particularly significant in transplantation.

5.1. Impact of weight difference on primary graft function

Poor early graft function (EGF) including delayed or slow graft function (DGF or SGF) is a frequent complication (2%-50%) after kidney transplantation particularly after cadaveric kidney transplantation (43, 120). An earlier identification of DGF and its associated factors is required, which would confer an important advantage. Indeed, experimental research on renal transplantation manifested the importance of day 1 post-transplant in evaluating serum creatinine and renal biopsy. Electron micrographs revealed evident disruption of tubular cells of kidney isograft with poor early graft function on day 1 post-transplantation (121). Acute tubular necrosis developing during reperfusion mainly caused DGF (43). On the other hand, DGF diagnosis at an earlier timepoint can facilitate a better therapeutic treatment strategy (120). Our present study revealed a significant relationship between body weight differences and primary graft function (on day 1) in a rat transplant model.

Although clinical studies have previously shown that body weight differences between donor and recipient can affect early graft function, the underlying molecular mechanisms and treatment options are unknown. Here, we investigated how transplanting kidney allografts from donors with reduced body weight affects early graft function, kidney morphology and gene expression. We could show that allografts from H-WD recipients are characterized by an increase in tubular HO-1 and interleukin 6 expression (Figures 6 and 7). The enhanced interleukin 6 expression may be the reason for the delayed graft function, morphological and molecular alterations as targeting interleukin 6 signaling could completely reverse these findings.

Our findings that transplanting kidneys from donors with reduced body weight results in impaired graft function is consistent with previous reports from experimental and clinical transplantation (39-42, 122, 123). An insufficient nephron mass fails to meet the metabolic demands of the kidney recipient resulting in glomerular hyperfiltration and hypertension (48), which is also observed after operational reduction in renal mass (124). Glomerular hypertension results in mechanical damage to the capillary wall and increased filtration of proteins to tubular lumen (125). The increased metabolic demand in tubular cells, as observed in case of glomerular hyperfiltration, leads to relative hypoxia (126) and synthesis of proinflammatory cytokines (125) resulting in tubular injury and cell death (124). Indeed, data presented here and by other groups demonstrate massive tubular necrosis in situations of reduced renal mass (124). In the case of transplanting a donor kidney with reduced nephron mass in relation to the recipient's demand, the tubular injury will be further exacerbated as reactive oxygen species (ROS) are produced after reoxygenation following ischemia / reperfusion (127).

The detected tubular damage was associated with increased interleukin 6 (IL-6) and Heme oxygenase 1 (HO-1) mRNA (Figure 6) and protein (Figure 7) expression within the tubuli of H-WD kidneys. HO-1 and IL-6 are induced by various stimuli, including pro-inflammatory cytokines/endotoxin, ischemia/reperfusion, and other pathophysiological responses that induce oxidative stress (128-130). They are expressed in the renal proximal tubular epithelial cells upon activation, which play a major role in renal injury and dysfunction (131, 132). HO-1 is a critical factor induced rapidly in RPTEC by various oxidative stimuli in an effort to resist injury and serves as a protective gene (133). An insufficient nephron mass in the case of transplanting kidney grafts from donors with reduced body weight induces hypoxic stress of tubular epithelial cells, which results in HO-1 up-regulation. The induced HO-1 production confers cytoprotection through the generation of CO and the antioxidant bilirubin. They exert their effects by inhibiting the production of growth factors and cytokines and suppressing cell apoptosis (134). In the presence of induced HO-1, renal tubules may have the capacity to attenuate tissue damage induced by nephron mass insufficiency. Indeed, it has been shown that renal tubuli are dependent on intrinsic HO-1 production for their survival under oxidative stress (132). Deficiency in heme oxygenase 1 has been shown to result in impaired renal hemodynamics and

exaggerates systemic inflammatory responses to renal ischemia (135). However, in our setting, stress-induced HO-1 up-regulation occurs to be late as it can only confer protection after transplantation and ischemia / reperfusion when the recipients are preconditioned (136).

As mentioned above, H-WD kidneys with impaired primary graft function were also characterized by increased IL-6 mRNA and protein expression. Up-regulation of IL-6 has been frequently observed in biopsy and urine samples of patients suffering from delayed graft function (137, 138). It has been also reported that high pre-transplant soluble IL-6 receptor levels are associated with an increased risk of developing acute tubular necrosis (43). An increased production of IL-6 by RPTEC was detected *in vitro* in response to many diverse stimuli (139). After reperfusion gene expressions of IL-2, IL-6, and IL-10 were significantly up-regulated in renal tubules compared to biopsies taken after cold ischemia (140). Furthermore, the amount of IL-6 produced correlated with the degree of ischemic injury (141, 142). The increase of inflammatory response is important for further stimulation of the immune response which will affect long-term kidney graft survival. Moreover, high plasma levels of IL-6 and urinary IL-6 concentrations have been found to be associated with acute rejection episodes (43, 143). A more recent report showed that IL-6-deficient grafts transplanted into allogeneic wild-type recipients have significantly prolonged survival, approximately three times the survival time of wild-type controls (144), indicating a critical role for graft-produced IL-6 in allograft rejection. However, previous experimental data did not reveal the effects of weight difference between donor and recipient on renal tubular epithelial cell IL-6 production. Here we report remarkably augmented IL-6 production in tubuli of H-WD kidneys.

The increased IL-6 production in H-WD kidneys implied a pathophysiological role in tubular injury due to insufficient nephron mass. Therefore, we tested whether targeting IL-6 signalling by applying a neutralising anti-IL6R antibody immediately after transplantation will rescue primary graft function. Impact on primary graft function was compared to a standard prednisolone treatment. Steroid treatment of H-WD recipients prevented intragraft up-regulation of HO-1 and IL-6 (data not shown). Glucocorticoids are capable of inhibiting the induction of IL-6 mRNA and protein expression. The molecular mechanism by which glucocorticoids inhibit IL-6 gene expression has previously been proven to include glucocorticoid response elements

within the IL-6 promoter (145). Moreover, the increase of serum IL-6 level due to acute rejection episodes in renal transplant patients can be reduced rapidly and remarkably by administration of glucocorticoids (103). Although glucocorticoid treatment could restore primary graft function as measured by serum creatinine and inhibited IL-6 and HO-1 induction, tubular injury (Figure 9) could not be completely prevented. As induction of IL-6 occurs very early in many models of acute kidney injury (142, 146), inhibition of IL-6 expression by steroids might not prevent the inflammatory response of immediately produced IL-6. Anti-IL6R mAb treatment had no effect on expression of HO-1 and IL-6 in comparison to untreated H-WD grafts. However, targeting IL-6 signalling completely prevented tubular injury caused by the insufficient nephron mass (Figure 9) and restored primary graft function. A detrimental role for IL-6 has been described in models of acute kidney injury, ischemia / reperfusion injury and chronic inflammation (146-148). Furthermore, targeting IL-6 signalling can ameliorate renal injury caused by ischemia / reperfusion (147). Our findings indicate that targeting IL-6 signalling is a specific and superior treatment option in comparison to a rather unspecific glucocorticoid treatment with all known side effects.

We could show here that transplanting kidneys from donors with reduced body weight, reflecting a reduced nephron mass in relation to the recipient's metabolic demands, results in poor primary graft function associated with a massive tubular injury. IL-6, upregulated in tubular cells of such H-WD recipients, plays a critical role during this process. Immediate targeting of IL-6 signaling offers an effective and specific treatment option and might enable better management of transplant patients receiving grafts from weight-mismatched donors.

5.2. Impact of CMV on graft function

The phenomenon that previous infections cause the generation of T lymphocytes reactive to other antigens has been defined as heterologous immunity (31). In transplantation models, acute infection of cytomegalovirus (CMV) enhances the degree of vasculopathy and dramatically shortens the survival time of the allograft. Elimination of allogeneic responses could prevent cytomegalovirus-accelerated chronic rejection (149).

Moreover, it has been shown previously that RCMV infection can crossreact with an allo-specific immune response, which interferes with transplantation tolerance induced by the non-depleting anti-CD4 mAb, eventually leading to chronic allograft damage and decreased long-term graft survival (30). In our present study, both the Maastricht strain as well as the more infectious England strain RCMV at a low dose failed to interfere with allograft function and survival, whereas enhanced and accelerated chronic rejection was observed by RCMV infection at a high dose or when administered twice (Figure 11 and 12). The second CMV infection led to an activation of preexisting alloreactive memory T cells and severe T-cell responses (30). Although peripheral RCMV copies detected in the repeated infection group (G10) were comparable to the other groups (Figure 10), only repeated RCMV infection induced acute rejection episodes. These results indicate the danger of immune activation following cytomegalovirus infection, which seems to be more important than direct viral effects on the kidney transplant. Additionally, prolonged cold ischemia of the allograft was reported to aggravate accelerated chronic rejection by CMV infection in a rat renal transplant model (84). But our present data demonstrated that prolonged cold ischemia, the timing of contralateral nephrectomy and the use of different RCMV strains were no determinants of impaired allograft function after transplantation (Figures 11 and 12).

The allograft recipients died in the groups of Maastricht strain R-CMV infected at a high dose (G7) and repeated R-CMV infection (G10), indicating that a higher RCMV titer or repeated CMV infection could result in higher incidence of allograft rejection episodes and therefore increase the allospecific immune response leading to impaired long term graft function. Although England strain but not the Maastricht strain has the ability of efficient replication and invasion (150), we detected no differences regarding graft function or survival between recipients of these two RCMV strains. Thus, the timing, number and the dose of RCMV infection but not the strain itself seem to contribute to the effect of RCMV on transplant outcome.

Noticeably, it is important to monitor the production of longitudinal serum IgG allo-antibodies irrespective of early, mid-term or long-term detection. On day 5 post-Tx an increased production of circulating allo-antibodies suggested potential risk of oncoming graft dysfunction (Figure 19B). Nonetheless, the low level of serum IgG at early stage post-Tx cannot preclude grafts from succeeding acute rejection. Indeed,

based upon our observations, one G10 group recipient dying from an acute rejection on day 14 as evidenced by increased serum creatinine levels did not show any signs of elevated serum IgG allo-antibodies on day 3 or 5 post-Tx (Figure 19A). The aforementioned data imply that an elevated level of circulating donor-specific allo-antibodies on day 5 provides a risk factor for a potential rejection episode but is not a prerequisite. During the mid- or long-term follow-up after Tx (between day 60 and day 100), a sudden rise of serum IgG level may represent a high probability of a developing rejection. But further studies are required to validate and verify this conclusion.

Our study revealed that the allospecific immune response resulted in inhibition of activation-induced cell death of effector T cells (Figure 20). On the other hand, when inhibiting the immune response with an anti-CD4 mAb, $CD4^+$ but not $CD8^+$ T lymphocytes in the spleen became susceptible to apoptosis (Figure 20). Indeed, overexpression of Toag-1 could induce higher susceptibility to apoptosis in murine $CD4^+$ T cells (Keeren *et al.* Journal of Immunology-in revision). Accordingly, these observations imply that activation of an allospecific immune response can result in a decreased susceptibility of $CD4^+$ T cells to apoptosis probably via earlier down-regulation of Toag-1, which is detrimental to allograft survival.

Subsequently, to dissect the specific cellular immune responses of recipients at day 5 post-Tx, we next investigated the frequency of memory and effector T cells in PBLs. Our findings revealed that the frequency of memory $CD4^+CD45RC^{low}$ at day 5 post-Tx was elevated in the periphery by non-depleting anti-CD4 mAb treatment, accounting for peripheral tolerance induction (Figure 20). Conversely, allogeneic response reduced the frequencies of $CD4^+CD45RC^{low}$ T cells. The regulatory ability of $CD4^+CD45RC^{low}$ was proven previously. Selective transfusion of donor memory $CD4^+CD45RC^{low}$ T cells could greatly improve post-transplant immune reconstitution without risk of GvHD induction (116, 151). Thereby, increased frequency of $CD4^+CD45RC^{low}$ T cells might contribute to the induction of transplantation tolerance whereas decreased percentage of $CD4^+CD45RC^{low}$ T cells is detrimental to allograft survival. On the other hand, although the $CD8^+CD45RC^{low}$ T subset expressing increased levels of Foxp3 possesses suppressive activity for effector T cells rejecting transplanted organs and is able to inhibit the production of IFN-gamma by autoreactive T cells (152), our findings reveal that changes in the frequency of

$CD8^+CD45RC^{low}$ T cells in the periphery is not related to transplant tolerance induction in this model. However, in the spleen, the percentages of these subpopulations ($CD4^+CD45RC^{low}$ or $CD4^+CD45RC^{high}$ T cells) were similar in all groups, independent of whether they would develop acute rejection of untreated recipients or transplantation tolerance induced by anti-CD4 mAb.

5.3. Impact of exogenous IL-2 on graft function

As we have previously described (22), anti-CD4 treatment (RIB5/2) partially reduced intragraft cellular infiltration and prevented mononuclear cell activation by lack of IL-2 receptor expression and inhibition of IL-2 production. Consistent with previous findings (26, 35) our results show that exogenous IL-2 administration reversed anti-CD4-induced tolerance and led to remarkable monocyte/macrophage infiltration into the grafts, as evidenced by the increased amount of ED1 expression in the allografts of high-dose IL-2-treated recipients (Figure 26). Accumulating evidence suggests that exogenous IL-2 is partially able to reverse the immunosuppressive effect of interleukin-10 by up-regulating the cell-mediated alloreactive response (153). Moreover, addition of IL-2 is associated with an increased intragraft $CD4^+$ and $CD8^+$ T cell and NK cell infiltration into the transplanted grafts, resulting in allograft rejection, even in a cyclosporine-induced long term graft acceptance model (5, 154, 155). Although we observed no acute rejection in our study, high IL-2 treatment caused a more severe glomerusclerosis, indicating acceleration of chronic rejection (Figure 22).

It is widely accepted that proteinuria is a key indicator for the progression of tubulointerstitial injury. Tubulointerstitial damage with scarring is the final common pathologic pathway in many forms of chronic proteinuric renal diseases (156). However, in our present investigation, a high dose of exogenous IL-2 did not cause a significant increase of urine protein production (Figure 21).

In our study, the total excretion of creatinine was similar among three groups. But concentration of urine creatinine was reduced relative to the IL-2 dose applied (data not shown). Renal tubules are mainly responsible for urinary concentration regulating the reabsorption of sodium and water (157). Even a slight destruction of tubular function can be reflected by concentration of the urine (157). In particular, urinary concentrating ability of kidney is best illustrated by the amount of “solute-free” water

reabsorbed from the glomerular filtrate (158). Interestingly, our findings revealed that exogenous IL-2 administration had a significant effect on increasing urine volume in a dose-related response. Past studies proved that after partial nephrectomy, the glomerular filtrate rate (GFR) of the remnant kidney increases 3-fold. Although electrolytes were well-maintained all the time, the excretion of water was markedly augmented, as a sign of impaired of kidney function (159). Loss of renal mass damaged tubular function, noticeably in fractional proximal reabsorption (159). Therefore, our findings indicate an IL-2 mediated kidney graft injury, resulting in impaired urinary concentrating function and water reabsorption.

Naive $CD4^+$ T cells can develop into regulatory T cells by acquiring the expression of the IL-2 receptor alpha-chain (CD25) and the transcription factor Foxp3. Indeed, Foxp3-knock-out mice lack regulatory T cells and develop fatal autoimmune pathology (160). Therefore, it was interesting to analyze the gene expression of CD25 and Foxp3 in the peripheral blood and compare the relative frequencies of peripheral $CD4^+CD25^+Foxp3^-$ and $CD4^+CD25^+Foxp3^+$ T cells in our long term surviving recipients receiving either no treatment, a low dose IL-2 treatment or a high dose IL-2 treatment. Administration of exogenous IL-2 led to a transient increase of peripheral CD25 and Foxp3 mRNA expression at the early stage (day 2 and day 5) (Figure 23). Moreover, significant elevation of relative and absolute numbers of $CD4^+CD25^+Foxp3^-$ T cells in the peripheral blood was observed in the high dose IL-2 treatment group (Figure 25). $CD4^+CD25^+Foxp3^-$ T cells functioning as effector T cells are able to produce IFN-gamma (161). Therefore, administration of exogenous IL-2 provoked early formation of adaptive immune responses. Subsequent production of $CD4^+CD25^+Foxp3^-$ effector T cells is ascribed to acceleration of chronic rejection.

Indeed, IL-2 signaling can promote differentiation of IFN-gamma-secreting effector T cells and regulate cytotoxic lymphocyte activity (162). The real-time PCR analysis of peripheral blood lymphocytes revealed an upregulation of Perforin transcription in the early phase by IL-2 (Figure 23), which may contribute to enhanced T cell alloreactive responses observed in previous studies (23). According to previous experimental observations, a high dose of exogenous IL-2 could restore allospecific immune responses that were inhibited by RIB5/2 (6). Moreover, further evidence from an in vitro model proved that anergized cells could de novo respond to alloantigen by use of exogenous IL-2 (163).

5.4. Prediction of long-term graft outcome under different conditions

In the two transplant models we studied (RCMV infection and IL-2 application), kinetic analysis of intragraft gene expression and/or peripheral gene expression revealed that transcript analysis of CD3, Perforin, Tregs-associated Foxp3 or costimulatory signal molecules including CD40L and CTLA-4 could not be used for distinguishing between tolerance induction, chronic or acute rejection. Nonetheless, serum IgG level, peripheral gene expression level of RHAMM and Toag-1, intragraft gene expression level of PDZK-1 and Toag-1 might be used for predicting graft outcome or monitoring allo-specific immune responses in the periphery or within the graft (Figures 16, 17, 18, 19 and 23).

Tregs mainly expressing CD25 play an important role in regulating immune homeostasis. However, the expression level of CD25 also reflects the degree of T cell activation. Therefore, it is reasonable to explain that intragraft allo-antigen-specific immune response sensitively caused a significant upregulation of CD25 mRNA expression in the local niche (Figure 15), which can be utilized to evaluate intragraft immune status. However, our data did not support that peripheral gene expression level of CD25 was associated with graft function (Figure 17), implying no direct relationship of CD25 expression between intragraft and in the periphery. This finding is consistent with the result that the frequency CD25-expressing T cells could not differentiate between acute rejection from tolerance induction (164). Interestingly, the degree of intragraft PDZK-1 mRNA expression down-regulation was also able to predict graft outcome despite the fact that there is no relation with intragraft immune responses. PDZK1 originally isolated from a kidney cDNA library possesses four well-defined PDZ-binding domains and was identified in kidney, liver, pancreas, gastrointestinal tract, and adrenal cortex, but not in the peripheral blood (109). We detected an intragraft down-regulation of PDZK-1 gene expression early after transplantation not only in samples of untreated recipients (Figure 16, G1) but also in samples of anti-CD4 mAb-treated recipients (G2) although the decrease was not as apparent. This directly resulted from inflammatory factors mediating ischemia/reperfusion injury (165), which predominantly affects proximal tubular epithelial cells (PTECs) of the kidney (166). PDZK-1 is exclusively localized in the brush border of PTECs. Additionally, PTECs are main targets during an acute rejection of kidney grafts (167). In untreated recipients, ischemia/reperfusion also

activates persistent renal and extrarenal immune responses (168), aggravating tubular cell injury (169), thereby leading to severe brush border damage accompanied by a dramatic decrease of PDZK1 expression. In tolerance developing recipients, PDZK1 gene expression de novo increased at day 14 post-Tx, suggesting functional recovery of renal epithelial tubular cells. On the other hand, although PDZK1 deficiency in an animal model manifested a profound defect in oxalate-stimulated NaCl absorption in the proximal tubule, abnormal plasma electrolytes were not found (109). Therefore, monitoring plasma electrolytes instead of intragraft PDZK-1 gene expression would not be useful. Therefore, studying the degree of intragraft PDZK1 downregulation could be utilized as a marker of acute rejection, particularly as a potential indicator of the deterioration of graft function.

As a non-invasive marker, peripheral gene expression level of RHAMM may be utilized to monitor graft dysfunction. In the exogenous IL-2 tolerance abrogation model, RHAMM gene expression in PBLs could not precisely differentiate between tolerance induction and tolerance abrogation by IL-2 (Figure 23), but our study proved allogeneic responses might cause a significant increase of RHAMM transcript in the peripheral blood. Furthermore, although RHAMM gene expression in PBLs could not precisely predict graft outcome in the RCMV infection model, the transcript level of RHAMM in PBLs on day 5 could discriminate rejecting recipients from tolerance developing recipients (Figure 17). Moreover, RHAMM transcription was elevated in the peripheral blood when applying a high dose of IL-2, suggesting its potential role in predicting graft outcome (Figure 23). In fact, RHAMM is capable of regulating cell locomotion of macrophages and B and T lymphocytes (170). In our studies, the allograft as a foreign antigen activates the recipient's immune system, leading to re-distribution of immune cells particularly in the periphery. Thereby, the increase of RHAMM mRNA expression would facilitate cell motility and migration of macrophages and lymphocytes from peripheral blood into the graft reflecting the allo-immune response after transplantation.

Intriguingly, Toag-1 gene expression level either in PBLs or within the graft could precisely predict long-term allograft deterioration in both models. In the RCMV infection model, downregulation of intragraft or peripheral Toag-1 mRNA expression on day 5 was able to differentiate acutely and chronically rejecting recipients from recipients with no impact on tolerance induction. Moreover, Toag-1 transcript levels

on day 5 negatively correlated with recipients' urine protein level on day 150 post-Tx or the severity of glomerulosclerosis of transplanted allografts (Figure 18). In the RCMV infection model, different doses of RCMV could interfere with anti-CD4-induced tolerance, which was precisely prognosed by Toag-1 mRNA expression patterns on day 5 (Figure 17). Importantly, these findings were in line with not only their long-term graft outcome but also intragraft Toag-1 gene expression profile (Figure 16). In addition, it was also observed that despite normal serum creatinine level at day 3 in the group G1 a significant decrease of peripheral Toag-1 transcript level could already be detected earlier than impaired kidney function (Figure 17). Interestingly, synchronous alterations of Toag-1 gene expression between PBLs and grafts were observed, which provides possibilities for a kinetic peripheral monitoring of intragraft destruction as a non-invasive marker. In the IL-2 tolerance abrogation model, Toag-1 gene expression profile in PBLs also allowed prediction of graft outcome. Downregulation of Toag-1 mRNA expression level on day 5 post-administration of exogenous IL-2 was inversely correlated with the increase of urine output and extent of intragraft glomerusclerosis.

The underlying mechanism of intragraft Toag-1 mRNA expression in predicting long-term graft outcome has been partially explored before. Downregulation of intragraft Toag-1 expression is a result of activation of graft infiltrating cells (GICs) especially T cells (20). Serial analysis of gene expression change of Toag-1 provides mechanistic insights into the in vivo activity of the immune system. The presence of CD4+CD25+ regulatory T cells inhibiting T-cell activation resulted in a heightened level of Toag-1 expression (20). Therefore, intragraft Toag-1 mRNA expression level is a sensitive marker to reflect allospecific intragraft immune responses.

To further explore the mechanism of peripheral Toag-1 mRNA expression in predicting long-term graft outcome, analysis of T cell subset in the periphery and in the spleen was performed. During the formation of stable tolerance to MHC-incompatible allografts, apoptosis is often required to reduce alloreactivation, particularly of effector T cells (115). Therefore, in order to address the question whether T cell activation is correlated with a decreased percentage of peripheral T cells undergoing apoptosis, further flow cytometric analyses were performed. Anti-CD4 mAb treatment caused remarkable elevation of $CD4^{+}$Annexin_V^{+} cells in the spleen, indicating high susceptibility of $CD4^{+}$ T cells to apoptosis. This was closely

associated with early and concurrent downregulation of Toag-1 (Figure 20). However, the frequency of $CD8^+$Annexin_V^+ T cells was not significantly influenced. On the other hand, allo-specific immune responses in untreated acutely rejecting recipients reduced the frequency of $CD4^+$Annexin_V^+ T cells, inhibiting apoptosis of effector $CD4^+CD25^+Foxp3^-$ T cells in the peripheral blood (Figure 25). Accordingly, these observations demonstrate that initiation of an alloresponse could decrease susceptibility of CD4+ T cells to apoptosis via earlier down-regulation of Toag-1 expression, which is detrimental to allograft survival.

Indeed, it was revealed that Toag-1 protein exclusively expressed in the cellular mitochondria is dramatically decreased after T cell activation in vitro. Over-expression of Toag-1 led to higher susceptibility to apoptosis (Keeren *et al.*, JI in revision). Therefore, it is reasonable to explain that the level of peripheral Toag-1 gene expression in the tolerated recipients was significantly higher than that in the acutely rejected recipients. Thus our results indicate that RCMV infection depending on the dose can interfere with anti-CD4 mAb-mediated transplant tolerance induction resulting in peripheral and intragraft downregulation of Toag-1 gene expression.

Additionally, our findings revealed that circulating donor-specific IgG as a marker of humoral immunity was in part associated with the development of graft impairment. An increased level of peripheral alloantibody on day 5 is a risk factor of a developing graft dysfunction, but not a prerequisite. Nonetheless, a high titer of serum IgG in the mid- and long-term observation period is associated with a subsequent chronic rejection. Indeed, despite the use of potent anti-T cell agents, antibody-mediated injury may cause a proportion of acute and chronic humoral rejections (171). Unlike depleting anti-CD4 treatment leading to a marked reduction of antibody response (119), non-depleting anti-CD4 mAb treatment could not reduce the production of serum antidonor IgG antibody (172). Moreover, our study documented a dramatic rise of donor-specific IgG level in one only anti-CD4 mAb treated recipient who 90 days later developed impaired graft function. However, further studies are required to validate and elucidate these results.

6. SUMMARY (IN ENGLISH AND GERMAN)

Transplanting kidneys from donors with reduced body weight, reflecting a reduced nephron mass in relation to the recipient's metabolic demands, results in poor primary graft function associated with a massive tubular injury. IL-6, up-regulated in tubular cells of such H-WD recipients, plays a critical role during this process. Immediate targeting of IL-6 signaling offers an effective and specific treatment option and might enable better management of transplant patients receiving grafts from weight mismatched donors.

RCMV infection and exogenous IL-2 administration interferes with transplant tolerance induced by non-depleting anti-CD4 mAb. The tolerance abrogation could be predicted by an early downregulation of Toag-1 gene expression in the peripheral blood on day 5 post-transplantation or post-injection of IL-2. Furthermore, this down-regulation was significantly correlated with recipients' urine protein level on day 150 or the severity of glomerulosclerosis of transplanted allografts. The combined assessment of potential markers such as Toag-1 and RHAMM may improve precise prediction of allograft outcome. The underlying mechanism is that initiation of allospecific immune responses inhibit the generation of peripheral regulatory $CD4^+CD45RC^{low}$ T cells and reduce the frequency of peripheral $CD4^+Annexin\_V^+$ T cells, inhibiting the apoptosis of effector $CD4^+CD25^+Foxp3^-$ T cells in the peripheral blood. The allospecific immune response results in down-regulation of Toag-1 expression, which decreases the susceptibility of $CD4^+$ T cells to apoptosis.

In addition, intragraft gene expression level of CD25 or PDZK-1 on day 5 posttransplantation might also be used for predicting graft outcome. Significant upregulation of CD25 and downregulation of PDZK-1 was observed in the rejected grafts. Interestingly, kinetic monitoring for serum allo-antibodies (IgG) is also significant. An elevated level of circulating donor-specific allo-antibodies on day 5 provides a risk factor for a potential rejection episode but is not a prerequisite. During the mid- or long-term follow-up after Tx (between day 60 and day 100), a sudden rise of serum IgG level indicates a high risk of a developing rejection. Nevertheless, further and extensive studies are required to validate our present findings.

Zusammenfassung

Die Transplantation der Nieren von Spendern mit gegenüber den metabolischen Anforderungen der Empfängertiere reduziertem Körpergewicht führte zu einer schlechten Primärfunktion verbunden mit einer massiven Schädigung der Tubuli. IL-6, produziert von den tubulären Epithelzellen der Empfängertiere, spielt eine große Rolle in diesem Prozess. Eine rechtzeitige Beeinflussung der IL-6 Signaltransduktion eröffnet eine effektive und spezifische Behandlungsmöglichkeit und damit ein besseres Management transplantierter Patienten, die eine Niere von gewichtsreduzierten Spendern erhalten.

Eine CMV Infektion oder die Gabe von IL-2 verhindert eine durch anti-CD4 Antikörper Behandlung induzierte Transplantattoleranz. Der Zusammenbruch der Toleranz kann anhand einer frühen Verringerung der Toag-1 Transkription im Blut am Tag 5 nach Transplantation oder 5 Tage nach der ersten IL-2-Behandlung vorhergesagt werden. Ebenso korrelierte das Ausmaß der Transkriptionsverringerung am Tag 5 nach Transplantation mit der Proteinurie und dem Schweregrad der Sklerose am Tag 150. Eine Kombination der Expressionsanalyse von Toag-1 und Rhamm scheint die Vorhersage der Langzeitfunktion noch zu verbessern. Untersuchungen zu zugrunde liegenden Mechanismen ergaben, dass die Initiierung der allo-spezifischen Immunantwort die Generierung regulatorischer $CD4^{+}CD45RC^{low}$ T-Zellen in der Peripherie verhindert und die Frequenz $CD4^{+}$Annexin_V^{+} T-Zellen Zellen reduziert und damit die Apoptose $CD4^{+}CD25^{+}Foxp3^{-}$ Effektor-T-Zellen verhindert. Die allo-spezifische Immunantwort bewirkt eine Verringerung der Toag-1 Transkription in aktivierten T-Zellen und somit eine verringerte Empfänglichkeit der T-Zellen gegenüber Apoptose.

Weiterhin zeigten die Untersuchungen, dass die Expressionsanalyse von CD25 und PDZK-1 im Transplantat am Tag 5 nach Transplantation auch zur Vorhersage der Langzeitfunktion geeignet ist. So konnte eine signifikante Erhöhung der CD25 und Verringerung der PDZK-1 Transkription nachgewiesen werden. Interessanterweise, ergab eine kontinuierliche Analyse der Allo-Antikörper im Serum signifikante Ergebnisse. Ein erhöhter Spiegel an Allo-Antikörpern am Tag 5 nach Transplantation stellt ein erhöhtes Risiko für die Entwicklung einer akuten Abstoßung dar, ist aber nicht zwingend notwendig. Während der plötzliche Anstieg der Allo-Antikörperspiegel

zu einem späteren Zeitpunkt (zwischen Tag 60 und 100) ein hohes Risiko für die Entwicklung einer chronischen Abstoßung darstellt. Trotz dieser viel versprechenden Ergebnisse sind weitere Untersuchungen zur Bestätigung unserer Daten erforderlich.

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8. ABBREVIATIONS

aCD4	anti-CD4 mAb treatment
AR	acute rejection
CD40L	CD40 ligand (CD154)
CMV	cytomegalovirus
CR	chronic rejection
CTLA-4	cytotoxic T lymphocyte-associated antigen-4
CXCL-13	CXC chemokine ligand-13 (B cell attracting chemokine-1)
DCs	dendritic cells
DST	donor specific transfusion
Foxp3	forkhead box P3
GFR	glomerular filtrate rate
GVHD	graft-versus-host disease
HCMV	human cytomegalovirus
HO-1	heme oxygenase-1
H-WD	High-weight difference
IE	immediate-early
IL-	interleukin
I.p.	intraperitoneally
KTx	kidney transplantation
L-WD	low-weight difference
mAb	monoclonal antibody
MCF	mean channel fluorescence

MHC	major histocompatibility complex
PBLs	peripheral blood leukocytes
PBS	phosphate-buffered saline
PDZK-1	PDZ binding kinase-1
PFU	plaque forming units
PTECs	proximal tubular tubular epithelial cells
RCMV	rat cytomegalovirus
RHAMM	a Receptor for Hyaluronan-Mediated Motility
RhoGAP	Rho GTPase Activating Protein
RT	room temperature
RT-PCR	real-time polymerase chain reaction
TA	transplant arteriosclerosis
TCR	T cell receptor
TI	Tolerance induction
Toag-1	tolerance associated gene-1
Tregs	regulatory T cells
Tx	transplantation

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10. CURRICULUM VITAE

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

11. PUBLICATIONS

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12. DECLARATION

I, Weihua Gong borne on 03/03/1977 in Zhejiang Province, P.R.China hereby declare that the dissertation entitled „Monitoring allograft acceptance and function in clinically relevant transplant models“ was finished by myself and without unauthorized help or information. I did not copy other works and a list of the references employed is included.