Rationale and effect of reduction of immunosuppressive load in organ transplant recipients

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Dr. C.C. Baan
Prof. Dr. R. Zietse.

Het is niet erg iets moois te verliezen, beter verliezen dan dat je nooit hebt gehad

Heilige Antonius

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Chapter 1

General introduction and outline of this thesis

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Giving a patient immunosuppressive medication is creating an environment in which a transplanted organ will be accepted and rejection will be prevented. Unfortunately, the use of immunosuppression is complicated by serious side effects. After dealing with acute rejection in solid organ transplantation and reducing the incidence of infections in the early days of transplantation, other serious complications became more and more clear. The development of, for example, cardiovascular disease, diabetes mellitus, nephrotoxicity and malignancies after solid organ transplantation is a well known problem for every transplant clinician.

Cardio vascular disease

For many years now, cardiovascular disease is the leading cause of death with a functioning graft and the most common cause of transplant failure.

Many chronic renal disease patients already have a history of cardiovascular disease before transplantation and the prevalence of the traditional risk factors for cardiovascular complications is high in this patient group (1-2). On top of this, patients suffer from non-traditional risk factors related to poor kidney function such as altered calcium-phosphate metabolism, hyperparathyroidism, chronic inflammation, anaemia, microalbuminuria, homocysteinemia and volume overload (3-4). Moreover, hypertension, diabetes, dyslipidemia, obesity, CMV infection and hyperhomocysteinemia are risk factors for cardiovascular disease which are exacerbated by the use of immunosuppression after transplantation (5)-(6).

So, the reported mortality rate of cardiovascular disease in patients with end stage renal disease (ESRD) is between 10 till 20 times higher then in the general population (4, 7). The mortality rate increases with duration of dialysis, but even after transplantation it remains high (4, 7-8). Ojo et al, described in 2006 an annual risk of a fatal or non-fatal CVD event of 3.5 to 5% in kidney transplant recipients which is 50-fold higher than the general population(9).

Malignancies

In all reports from different sides of the world, the incidence of almost all tumour types, skin and non-skin, is increased after solid organ transplantation compared with the general population. Cancer is currently the second or third highest cause of death in renal transplant recipients (10-12). Skin cancer is the most commonly observed type of de novo malignancy after transplantation with a reversed ratio of basal cell carcinoma (BCC) to squamous cell carcinoma (SCC) compared with the general population, in which basal cell carcinoma is the most common (13-16). Given the relationship between sun exposure and skin malignancies, it is not surprising that the incidence of skin malignancies in the Australian and New Zealand transplant population is high. Ramsay et al reported a cumulative incidence of skin cancer in the

Queensland renal transplant population of 82% after more than 20 years of exposure to immunosuppression (17). However, even in areas with less sun exposure, like the United Kingdom, the cumulative incidence is still high with numbers of 61% 20 years after renal transplantation, while 64% of the patients had multiple lesions(18). Studies in the Dutch and Norwegian transplant recipients, both populations with moderate sun exposure, showed that SCC occurred 65-250 times and BCC 10 times as frequently as in the general population (19-20) with a high risk of subsequent non-melanoma skin cancer (21). Sixty-four to 74% of this transplanted patients with non-melanoma skin cancer had multiple lesions, with a maximum reported cumulative number of skin lesions of 50 in one patient. Almost 4% of the patients who developed a non-melanoma skin cancer died due to metastatic disease (21).
As with skin cancer, transplanted patients are at elevated risk for almost all de novo solid tumor types. Overall, the standardised incidence ratio (SIR), defined as the ratio of the observed number of tumors in the transplanted population to the expected number of tumors in the general population, matched for age, gender and occurring in the same calendar year, is approximately 2.5-4 times higher (22-24). The cumulative risk of developing at least one malignancy (excluding nonmelanoma skin cancer) while the graft continued to function reaches 30% after 20 years. The relative risk varies by cancer site, with some risk on solid tumor types only moderately and others seriously elevated compared with the general population, depending on age, gender, race, primary cause of ESKD, racial background, prior malignancy and continued graft function. This excess relative risk is not constant but is inversely related to age: the greatest relative risk is experienced by younger recipients of both sexes. For example, an analysis of the Australian and New Zeeland population by Webster et al showed us a cancer rate for a 25 year-old female renal transplant recipient equivalent to that of a 55 year-old woman in the general population. With increasing age, this risk declines towards that experienced by the general population, although the risk for developing cancer for recipients over the age of 65 years is still 2-3 times above that of the general population(23). This results in a different distribution of malignancies in the transplanted population compared with the general population, and therefore not all existing screening programs are applicable in this patient group.

The pathogenesis of cancer in organ transplant recipients is complex because of the multiple pathogenetic factors in these patients.
The incidence of cancer increases over time and besides the known risk factors for cancer in the general population like age, gender, smoking habits, genetic predisposition, etc., there is interplay of several immunological and non-immunological factors after transplantation which increases the cancer risk in this specific population. For instance, the overall or cumulative exposure to immunosuppressive agents is closely correlated with the cancer risk. The use of immunosuppression disrupts antitumor

immunosurveillance and anti viral activity, but some agents promote carcinogenesis independent of their immunosuppressive effects (25-28) and/or may potentiate the carcinogenic effects of other agents (29). To prevent rejection, transplanted patients uses combinations of different immunosuppressive drugs. This makes it is difficult to asses the impact on cancer risk of each individual immunosuppressive agent. However, among non-transplant patients who use one specific immunosuppressive agent it has been described that, prednisone increases the risk of developing SCC, BCC, Kaposi sarcoma and non-Hodgkin lymphoma (NHL)(30-33). This is not an uniform finding: others reported that, in patients with polymyalgia rheumatica and temporal arteritis treated with high cumulative doses corticosteroids, this risk on NHL was not increased (34). Patients with psoriasis treated with cyclosporine have an increased risk on non-melanoma skin cancer (NMSC), especially SCC, as well as NHL(35-37). The use of azathioprine in patients with multiple sclerosis and rheumatoid arthritis is associated with a progressive rise in their risk on malignancy depending on the duration of treatment, although none of these increased risks were statistically significant.(38-39). A meta-analysis has demonstrated an increased risk on lymphoma in patients with inflammatory bowel disease treated with azathioprine or mercaptopurine(40). So it seems that immunosuppression per se results in a higher incidence of cancer.

An increased susceptibility to (viral) infections after transplantation, also implicates an increased risk to some malignancies. Certain viral infections clearly are linked with post transplant malignancies, including Epstein Barr virus (EBV) with PTLD(41-42), hepatitis B virus and hepatitis C virus with hepatocellular carcinoma (43-45), human herpes virus-8 with Kaposi's sarcoma(46-48) and papillomaviruses with SCC, oropharyngeal carcinoma and cervical cancer(49-51).

Another possible predisposing factor for developing cancer may be prolonged uremic status before transplantation and with graft failure. Chronic uraemia may be considered as a state of immunodeficiency. In particular, lymphomas and carcinomas of the kidney, bladder, prostate, liver and uterus show an enhanced prevalence in patients with impaired renal function, compared with the general population (52-54). Finally, the chronic antigen stimulation by the transplanted organ, combined with an inadequate cytotoxic T cell activity due to the chronic use of immunosuppression, may also represent an important cause of the tendency to form malignancies after transplantation.

Considering the above observations, minimization of immunosuppression whenever possible, as a strategy to reduce the incidence of post transplant malignances, seems to be warranted. This thought is supported by the fact that the risk of de novo cancers, which are not related to ESRD, like thyroid, bladder and urinary tract, returns to pretransplant levels after graft failure and return to dialysis (55).

Nephrotoxicity

The clinical introduction of the calcineurin inhibitors (CNI) cyclosporine (CsA) and tacrolimus (Tac) has dramatically reduced the incidence of acute rejection and thus improved the short time graft survival. However, the long-term results have not improved to a similar degree over the last few decades(56). An important contributing factor of this later observation is CNI induced nephrotoxicity. Nephrotoxicity after solid organ transplantation, but also in patients treated with CNIs for autoimmune diseases, can be distinguished in an acute and chronic form. The acute form of CNI induced nephrotoxicity was first described by Calne et al in the first publications of the clinical use of cyclosporine in human renal transplant recipients (57-58), whereas prior animal studies had not observed this important side effect (59-61). Acute CNI induced nephrotoxicity may manifest with variable severity and is characterised by a rise in serum creatinine levels with or without an oligoanuric syndrome. It usually starts several days after the introduction of CNI's. Pathophysiologically it is characterised by vasoconstriction of the afferent glomerular arteriole, leading to a decreased perfusion of the corresponding glomeruli and finally a decrease in glomerular filtration rate (GFR)(Figure 1)(62). Non-specific morphological tubular abnormalities also characterize acute CsA nephrotoxicity, including giant mitochondria, isometric vacuolization and microcalcification.
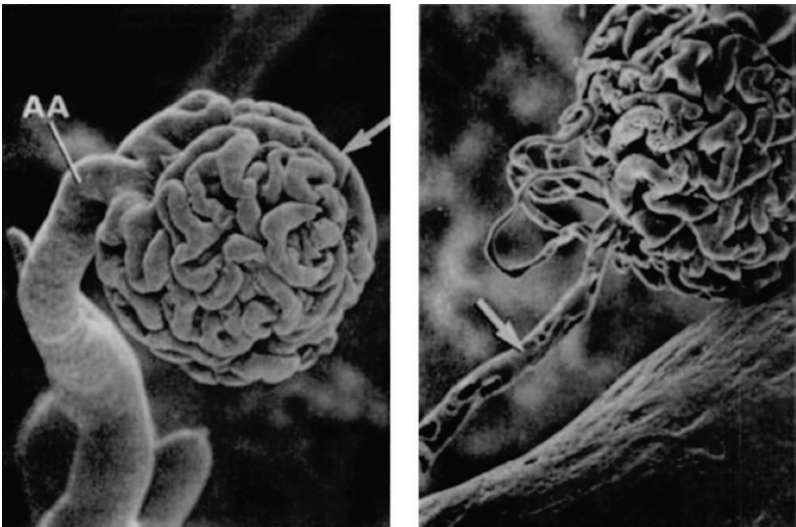
Figure 1.
(Left) Scanning electron micrograph of an afferent arteriole (AA) and glomerular tuft from a control animal.
(Right) From a similar animal after 14 d of cyclosporine treatment.

Reprinted from English J, Evan A, Houghton DC, Bennett WM: Cyclosporine-induced acute renal dysfunction in the rat: Evidence of arteriolar vasoconstriction with preservation of tubular function. Transplantation 44(1): 135–141, 1987 (reference 29), with permission.

The giant mitochondria tend to predominate in the convoluted section of the proximal tubule, whereas isometric vacuolization is mainly limited to the thick descending limb of the loop of Henle. More severe nephrotoxicity is often associated with arteriolopathy, characterised by focal myosite necrosis in the media of small arteries, in the absence of intimal changes, or trombotic microangiopathy. These changes are not pathognomonic for CNI induced nephrotoxicity, although they are particularly frequent in patients treated with CsA or Tac, and can be focal or even absent in the presence of clinical nephrotoxicity (63). For most patients, both the morphologic and functional changes are rapidly and completely reversible when dose reduction or withdrawal of the CNI has taken place (64-66). If not, other causes for renal dysfunction, like acute rejection, need to be excluded (67).

In 1984, Myers et al, were the first to describe an irreversible renal functional deterioration as a result of the long-term use of cyclosporine in heart transplant recipients, called "chronic CNI nephrotoxicity" (68). This chronic CNI induced nephrotoxicity is characterised by a slow, progressive decline of renal function, which may progress to end stage renal disease, and sometimes mild to moderate proteinuria. In addition most patients have hypertension. Later, these nephrotoxic effects of long-term use of cyclosporine and tacrolimus were confirmed by many others, and has been seen after all types of transplantation (69-72), but also after chronic treatment with CNI for auto-immune diseases (73-75). In 2003. Nankivell et al described a cohort of kidney-pancreas transplant recipients, treated with CsA, which they prospectively followed for up to 10 years after transplantation. Protocol kidney biopsies were obtained regularly after transplantation. Ten years after transplantation, the cumulative incidence of histological changes in the kidney, indicative of chronic CNI related nephrotoxicity, was nearly 100%(76). Although this result is impressive, we should note that this study did not contain a control group, so the influence of other causes like aging, hypertension etc. remains unknown. In 2002 Bagnis et al described a group of patients treated with CsA for uveitis. Among the 41 patients, the GFR decreased from 102 mL/min/1.73m^2 at the start of treatment to 88 mL/min/1.73 m^2 after 2 years of CsA therapy. Renal biopsies, taken before and 2 years after starting treatment with CsA, showed important histological changes over time with significant increases in glomerular sclerosis, thickening of Bowman's capsule, and tubular atrophy and interstitial fibrosis(77).

Histologically, al parts of the kidney can be affected by CsA or Tac treatment. Diagnostic pathological criteria for chronic CNI nephrotoxicity include arteriolar hyalinosis, glomerular sclerosis and thickening of Bowman's capsule, tubular atrophy (TA), and interstitial (striped) fibrosis (78-80). However, the non-specificity of most of these lesions makes the differential diagnosis very difficult and includes, for example, aging, diabetes mellitus, hypertension and, in renal transplantation, pre-existing donor injury or chronic (humoral) rejection. Besides this differential diagnosis, these are all conditions which may coexist with chronic CNI nephrotoxicity (80). The mechanisms

responsible for this chronic nephropathy are not completely elucidated. A combination of hemodynamic changes leading to ischemia, direct toxic effects of CNI on the tubules, and an increased expression of the profibrotic transforming growth factor-ß (TGF- ß) are considered important etiologic factors. TGF- ß expression of tubular cells is directly upregulated by CsA as well as by Tac (81-86). TGF- ß promotes interstitial fibrosis by decreasing the degradation and increasing the production of extracellular matrix proteins (87-88). In addition, TGF- ß induces epithelial mesenchymal transition, in which renal tubular epithelial cells lose their epithelial phenotype and acquire new characteristic features of mesenchyme. This transition is recognized as a major mechanism contributing to renal interstitial fibrosis (89-93). Although the persistent use and a higher doses of CNIs seems to be contributing factors to chronic CNI nephrotoxicity (94-95), there are recent studies who describe an individual susceptibility to CNI nephrotoxicity, depending on individual variation of the organ donor or recipient in drug transporters (for example ABCB1, formerly known as permeability glycoprotein or P-gp), drug-metabolizing enzymes (for example CYP3A) or other polymorphically expressed genes, such as, TGF- ß(96-97), vascular endothelial growth factor(98), and caveolin-1(99).

In contrast with acute CNI nephrotoxicity, renal function and histological changes in chronic CNI nephrotoxicity improves only little, if at all, after minimization or withdrawal of CNIs. So, better insight in the mechanisms responsible for CNI nephrotoxicity may guide us to develop preventing strategies for CNI toxicity.

Despite the negative aspects of organ transplantation mentioned above, renal transplantation is still the treatment of choice for most patients with ESRD, as it results in better patient survival and quality of live compared with dialysis (100). Given this knowledge, we have to search for an opportunity to minimize the immunosuppressive load to prevent long term complications and to improve patient's quality of live, although an adequate combination of various kinds of immunosuppressive drugs is needed to prevent acute rejection in the early post-transplant period. To assess at what time after transplantation immunosuppression can be safely reduced or stopped, the use of a proper biomarker that measures antidonor reactivity is helpful. For example, van der Mast et al and van Besouw et al have shown that conversion from CNI to mycophenolate mofetil (MMF) or azathioprine(AZA) in stable renal transplant recipients is a safe procedure when helper T-cell reactivity and donor specific cytotoxic T-lymphocyte precursor frequency is low (101-102). Besides the identification of patient's who can be weaned of immunosuppression, biomarkers could help us to predict and diagnose acute rejection before irreversible injury has occurred during these weaning protocols which would make these protocols easer to perform and ethically more acceptable. However, to understand more which mechanism leads to tolerance we need to study the influence of immunosuppressive medication on both immune activation cascades as in the immune suppressive counter mechanisms.

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Aim of this thesis

In this thesis, the incidence of long-term side effects, such as CNI induced renal insufficiency and malignancies, and the influence of these complications on patient and graft survival in Dutch heart and renal transplant recipients were analysed. Knowing the negative influence of immunosuppression on these complications, we studied the possibilities to taper the immunosuppressive load after transplantation and analysed the effects of this minimization in stable renal transplant recipients on both clinical and immunological parameters. This knowledge will help us understand the influence of immunosuppressive medication on immunological processes leading to tolerance or rejection of the graft which will help us to develop a more efficient immunosuppressive treatment strategy which is tailored to individual patient characteristics.

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Chapter 2

The impact of TGF-ß1 gene polymorphism on end stage renal failure after heart transplantation

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Abstract

Background: Nephrotoxicity is a major side effect of calcineurin inhibitors (CNI). Earlier we reported 8% of our heart transplant recipients reaching end-stage renal failure (ESRF). Now, with an extended follow up of 20 years, we re-evaluated the development of ESRF and studied its influence on survival and the impact of polymorphisms in codon 10 and 25 of the promoter region of TGF-ß on the risk of ESRF.

Methods: 465 patients were transplanted between 6/84 and 6/05. All were on maintenance CNI treatment. Development of ESRF was studied in the 402/465 (86.5%) patients surviving at least one year. Their median follow up was 8 years, total observation time of 3414 years. TGF-ß polymorphisms in codon 10 (Leu to Pro) and codon 25 (Arg to Pro) were analyzed with real time PCR in a cohort of 237 patients, with an observation time of 2329 years.

Results: Ten years survival of patients surviving at least one year was 58.5%. Seventy-three patients (18.2%) developed ESRF. Dialysis free survival was 60% at 15 years.

The RR for ESRF in Pro[10] carriers was 2.9 (CI 1.5-5.8) compared to patients with the Leu/Leu[10] genotype (p=0.002), while Pro[25] carriers had a RR of 2.6 (CI 1.4-4.8) compared to the Arg/Arg[25] genotype (p=0.002).

Survival of patients with ESRF was 1.5 years (median).

Conclusion: We found a highly significant association between TGF-ß polymorphisms and CNI induced ESRF after heart transplantation (HTx). Pro carriers of either codon 10 or 25 had a 2.6 to 2.9 times increased risk of developing ESRF. As ESRF after HTx results in high mortality rates these patients should no longer receive CNI-based immunosuppression.

Introduction

With improved results and longer survival after heart transplantation, we are confronted with the complications of long-term use of immunosuppressive medication. After the introduction of calcineurin inhibitors (CNI), the median survival of heart transplant recipients now exceeds 10 years(1). Apart from complications of immunosuppression in general, chronic treatment with CNI's more specifically may result in renal failure. The prevalence of renal dysfunction in heart transplant recipients, varies from 0 to 20 % in different studies (2-12). The disparity in reported prevalence's is due to differences in the definition of renal failure as well as in the duration of follow-up after transplantation in the different studies. Identification of patients at risk for renal dysfunction or finally end stage renal failure (ESRF) could contribute to prevention of this complication by adjustment of the therapeutic regime. Possible risk factors for the development or progression of renal dysfunction after heart transplantation such as older age at heart transplantation (HTx), pre- and post-transplant hypertension, pre- and post-transplant diabetes mellitus, male gender, immediate post HTx renal function have been mentioned(13-15).

Independent of the primary cause of renal dysfunction, fibrosis is a typical histological feature of progression to ESRF and transforming growth factor- ß1 (TGF-ß1) seems to play a central role in this process (15). Because, TGF-ß1 levels are associated with gene polymorphisms in the TGF-ß1 promoter region, we wondered about the effect of TGF-ß1 gene polymorphisms on the risk for developing ESRF(16-18)

Now, with an extended follow of 20 years, we studied the development of the ultimate consequence of CNI nephrotoxicity: end stage renal failure (ESRF) and its influence on patient survival. Moreover we analyzed the impact of polymorphisms in codon 10 and 25 of the promoter region of TGF-ß1 on the risk of ESRF.

Materials and methods

Patients

Between June 1984 and June 2005, 468 heart transplantations were performed in 465 patients in our centre. Their clinical characteristics were as follows: 368 (79%) patients were male, 100 (21%) female; their median age was 50 (range 2-71); their primary heart disease was ischemic heart disease (IHD) in 244 (52%) patients, dilated cardiomyopathy in 201 (43%) patients and primary valvular disease or other in 23 (5%) patients. Only patients with a creatinine clearance of more then 30 ml/min were accepted for cardiac transplantation. Sixty-six patients had a follow-up of less than 1 year. Thirteen of them were recently transplanted, 53 patients died within this first year. The causes of death were primary graft failure in 19 (36%), acute rejection in 10 (19%), peri-operative complications in 7 (13%), infection in 8 (15%), malignancy in 5 (9%) and 4 (8%) patients died of various other causes. None of them had been on dialysis. Development of ESRF was studied in the 402/465 (86.5%) patients surviving at least one year. ESRF was defined as the need to start renal replacement therapy. Follow up of all patients was performed in our outpatient clinic. During these visits, clinical and laboratory data were collected. The diagnosis of renal failure due to calcineurin inhibitor toxicity was made by exclusion of other causes, mainly by ultrasonography and urine analysis. A renal biopsy was done in only 5 patients. Histology of all these biopsies showed marked CNI nephrotoxicity.

Immunosuppression

In 21 years of heart transplantation, several immunosuppressive regimens have been used in our heart transplantation centre. All were based on CNI with or without induction treatments with OKT3, ATG and anti-CD20 antibody (19). All patients used CNI as early post-operative and as maintenance CNI treatment in combination with azathioprine or mycophenolate mofetil and prednisone. The majority of patients used cyclosporine as CNI, but since the year 2000 tacrolimus was introduced in a small group of 21 patients. In none of the patients CNI's were discontinued.

TGF-ß1 gene polymorphisms

TGF-ß1 polymorphisms in codon 10 (Leu to Pro) and codon 25 (Arg to Pro) were analyzed in a cohort of 237 HTx recipients transplanted between June 84 and January 99.

DNA isolation, amplification and detection of TGF-ß1 gene polymorphisms were performed as described before (20). In brief, the studied TGF-ß1 polymorphisms (+869, Leu[10] → Pro and +915, Arg[25] → Pro) were determined by dot blot hybridization. Two biotinilated oligonucleotide probes were used to determine each polymorphism. Two ml PCR product was spotted onto Hybond™-N+ membrane (Amersham Pharmacia,

Buckinghamshire, UK) and treated with 0.5 mol/liter NaOH and 1.5 mol/liter NaCl for 5 minutes to separate double-stranded amplified DNA, followed by a neutralization step with 1.5 mol/liter NaCl and 0.5 mol/liter Tris, pH 7.5, for 1 minute. The membranes were baked in a microwave for 5 minutes, and DNA was immobilized onto the membranes by cross-linking with ultraviolet for 1 minute. Thereafter blots were incubated in 10 ml of hybridization buffer containing 5X SSC, 0.5X Denharts solution, 0.2 mol/liter EDTA, 0.5% sodium dodecyl sulphate (SDS), and 0.1 ml sonicated herring sperm (Promega, Madison, Wisconsin, USA) at 42.5°c for 30 minutes. We added 400 ng of specific biotinilated oligonucleotide probe and allowed it to hybridize for 90 minutes at 42.5oc. The membranes were washed twice with 5X SSC and 0.1% SDS at room temperature for 5 minutes, followed by stringency washing with 1X SSC and 0.1% SDS at 58°c (1869, Leu10 3 Pro) and 61°c (1915, Arg25 3 Pro) for 30 minutes. Before visualizing the hybridized probes, the membranes were washed in 0.15 mol/liter NaCl and 0.1 mol/liter Tris buffer, pH 7.5, for 1 minute and treated with 0.5% blocking agent (Roche Diagnostics, Almere, The Netherlands) for 30 minutes. Subsequently, the membranes were incubated with a streptavidine horseradish labeled peroxidase conjugate (Amersham Pharmacia) for 30 minutes at room temperature before detection by chemoluminiscence using the ECL™ system (Amersham Pharmacia).

We determined TGF-ß1 genotypes in 2 replicate experiments.

Statistical methods

Data for this study were obtained by retrospective patient chart analysis. Survival curves were made using the Kaplan-Meier method and the log-rank test was used to compare the survival rates. Continuous variables are reported as means ± SD and tested by Student's *t*-test. Data that did not follow a normal distribution are presented as medians and tested by Mann-Whitney U test. Qualitative variables are reported as percentages and were tested by the Pearson's chi-squared test.

Potential associations with dialysis free survival and with patient survival were studied by means of univariable analysis and the multivariable Cox proportional hazards analysis. Dialysis free survival was censored for death; patient survival was censored for ESRF. Variables included in this study were: age, gender, primary heart disease, serum creatinine levels before transplantation, total cholesterol and triglycerides at one year, cyclosporine levels at one year, coronary artery disease at one year and TGF-ß1 polymorphisms in codon 10 and codon 25. The SSPS statistical package version 12.0.1 was used. P-values <0.05 were considered significant

Results

The median follow up of the 402 patients with at least one year follow up was 8 (range 1-20) years with a total observation time of 3.414 years. The mean serum creatinine level of the study group just before and one year after HTx was 107±31 and 139±46 µmol/l respectively. After Htx 72% of the patients developed hypertension, which was treated with calcium-antagonists for initial therapy. Patients with treated hypertension were equally divided between the group with and without ESRF.

At the time of observation, 73 of 402 (18.2%) patients with more than one year follow-up developed ESRF. The median time after HTx of developing ESRF was 92 months (range 11-239). Sixty percent of these 402 heart transplant patients were free from dialysis after 15 years (figure 1). The mode of renal replacement therapy was haemodialysis in 37 patients, peritoneal dialysis in 28 patients, unknown in 6 patients and 2 patients died just before dialysis could be started. Seven patients (4 of a deceased and 3 of a living donor) received a kidney transplant after they started dialysis. At time of observation 33 (89%) of the haemodialysis, 24 (86%) of the peritoneal dialysis patients and 5 (71%) of the kidney transplant recipient's had died.

No differences in age, gender, primary heart disease, serum creatinine level, lipid profile or diabetes mellitus pre HTx were found between patients who did (ESRF +) or did not (ESRF -) develop ESRF (table 1). However, cyclosporine through levels were significantly lower in the ESRF + group.

In the univariable analysis and the multivariable Cox proportional hazards analysis none of the previously mentioned factors significantly influenced the risk of developing ESRF.
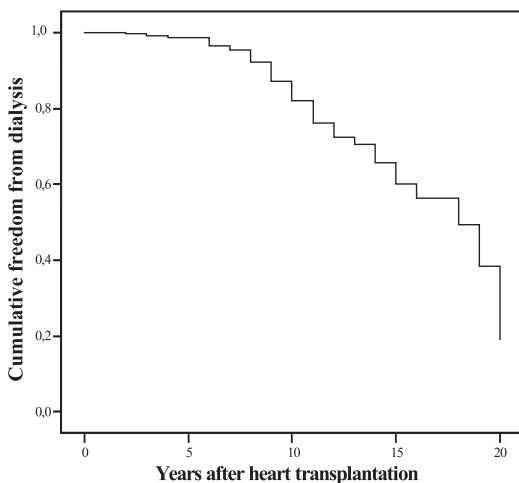

Figure 1. Kaplan-Meier curve of the cumulative freedom from dialysis censored for death in the one-year survivor heart transplantation group (n=402).

Table 1.

Demographics of patients with (ESRF +) and without (ESRF -) end stage renal failure after heart transplantation.

	ESRF +	ESRF -	p-value*
Number of patients	73 (18.2%)	329 (81.8%)	-
Male: Female	64:9	256:73	n.s.
Age (years, median, range)	49(15-64)	50 (4-71)	n.s.
Primary heart disease:			
-Ischemic heart disease	39 (53.4%)	167 (50.8%)	n.s.
-Cardiomyopathy	28 (38.4%)	147 (44.7%)	n.s.
-Valvular disease or other	6 (8.2%)	15 (4.6%)	n.s.
Creatinine level pre HTx (µmol/l, median, range)	105(69-232)	103 (36-282)	n.s.
CsA level at 1 year (ng/ml, median, range)	104 (45-420)	180 (31-610)	p=0.001
Triglycerides at 1 year (mmol/l, median, range)	2.22 (0.56-6.20)	2.11 (0.17-9.39)	n.s.
Total cholesterol at 1 year (nmol/l, median, range)	6.8 (2.6-10.9)	6.4 (1.1-15.5))	n.s.
Diabetes Mellitus pre HTx (n, %)	1 (1.4%)	13 (3.9%)	n.s.

* Mann Whitney test and Pearson's chi-squared test

The median patient survival time of the study group was 11.3 years (range 1–19.9). In the univariable analysis, increasing patient age at transplantation (p=<0.001), male gender (p=0.02), or presence of coronary artery disease at one year after transplantation (p=0.05) significantly influenced the death risk. Besides, primary heart disease significantly influenced this risk (p=0.003). In comparison to cardiomyopathy, ischemic heart disease had a relative risk (RR) of 1,6 (CI 1.2-2.2, p=0.002) and primary valvular disease had a RR of 2.2 (CI 1.2-4.1, p=0.013). Multivariable Cox proportional hazards analysis revealed that age was the only independent predictor of death (p=<0.001). There was a significant difference in the median survival between the ESRF + versus the ESRF- group, 9.50 versus 12.08 years respectively (p=0.0001). After reaching ESRF, the median survival time was only 1.5 years (range 0-14.7).

TGF-ß1 gene polymorphisms

The median follow up of the 237 patients of whom TGF-ß1 gene polymorphisms were analyzed was 9 years (range 1-20 years) with a total observation time of 2.329 years.

Table 2.

Distribution of TGF-ß genotypes between patients without (ESRF -) and with (ESRF +) ESRF for codon 10 and 25 in a cohort of 237 HTx recipients.

TGF-β genotype	ESRF - (n=180) n (%)	ESRF + (n=57) n (%)	
Codon 10			
Leu/Leu	79 (43.9)	10 (17.5)	
Leu/Pro	88 (48.9)	41 (72.0)	Leu/Leu vs Pro cariers
Pro/Pro	13 (7.2)	6 (10.5)	p=0.0003
Codon 25			
Arg/Arg	162 (90.0)	43 (75.4)	
Arg/Pro	16 (8.9)	14 (24.6)	Arg/Arg vs Pro cariers
Pro/Pro	2 (1.1)	0	p=0.008

The distribution of the TGF-ß1 gene polymorphisms, for codon 10 and 25 in patients with and without ESRF is shown in table 2. For codon 10, 82.5% in the ESRF + group versus 56.1% in the ESRF – group were Pro-carriers (p=0.0003). For codon 25, 24.6% in the ESRF + group versus 10% in the ESRF – group were Pro-carriers (p=0.008). None of the patients who were homozygote for leucine at codon 10, were pro-carriers at codon 25.

Fifty percent of the Pro[10] carriers were free from dialysis after 14.7 years compared to 19 years in patients with the Leu/Leu[10] genotype (p=0.001). Patients who were Pro[25] carriers, 50% are free from dialysis after 13.3 years versus 18.5 years in the patients with the Arg/Arg[25] genotype (p=0.01)(figure 2A and B).
In the univariable analysis both polymorphisms of TGF-ß1 thus were strongly associated with development of ESRF: Pro[10] carriers had a RR of 2.9 (CI 1.5-5.8, p=0.002) for developing ESRF compared to patients with the Leu/Leu10 genotype, while Pro[25] carriers had a RR of 2.6 (CI 1.4-4.8, p=0.002) compared to patients with the Arg/
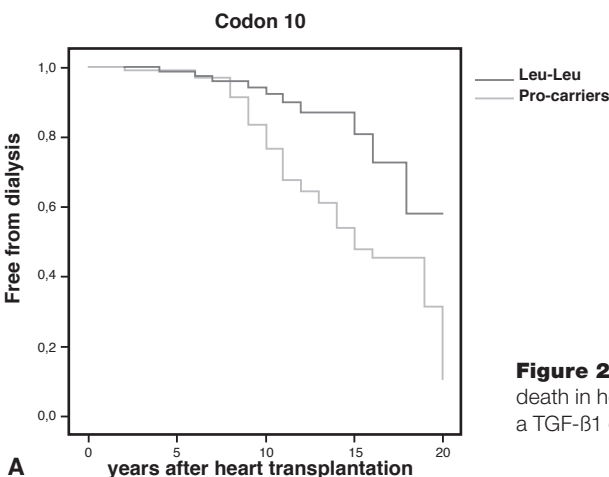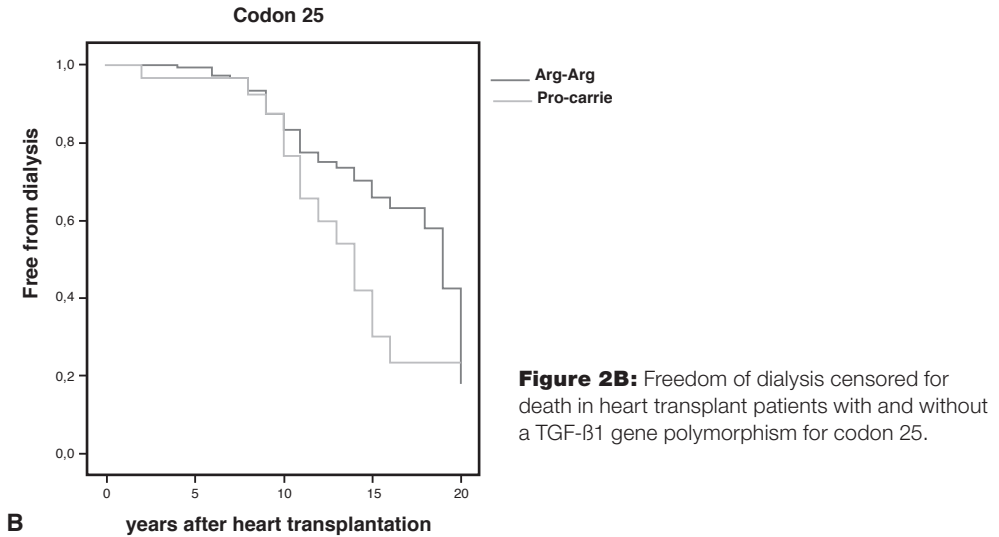

Figure 2A: Freedom of dialysis censored for death in heart transplant patients with and without a TGF-ß1 gene polymorphism for codon 10.

Codon 25

Figure 2B: Freedom of dialysis censored for death in heart transplant patients with and without a TGF-ß1 gene polymorphism for codon 25.

B

Arg[25] genotype. Multivariable Cox proportional hazards analysis revealed that only Pro[10] carriers (RR 3.4, CI 1.7-6.7, p=0.001) and older age per year (p=0.01) were independent predictors of developing ESRF after HTx.

Discussion

In this retrospective analysis, with an extended follow-up of 20 years, the prevalence of ESRF (18.2%) proved to be higher then the 8% reported earlier from our center (9). This reflects the problems that we face with longer patient survival after cardiac allograft transplantation and the associated long-term use of CNI.

In contrast with other studies, in our heart transplant recipients, no significant differences in age, gender, primary heart disease or lipid profile were found between the patients with and without ESRF(13-15).

Cyclosporine through levels were significantly lower in the ESRF + group, probably due to tapering of the CsA dose when renal dysfunction occurs. Indeed most studies in literature do not find a correlation between high CNI dose or through levels in patients who do develop ESRF compared with patients who don't (2-5, 7-12). This suggests an individually determined susceptibility for the nephrotoxic effect of CNI's. Factors responsible for CNI related nephrotoxicity are not completely understood. Immunohistochemistry has shown that kidneys with CNI induced morphologic abnormalities express the cytokine transforming growth factor TGF-ß1 (21-24). Furthermore, stimulation of the TGF-ß1 production in vivo after the use of CNI, has been described by several groups. So, TGF-ß1, with its profibrogenetic properties, seems to play a central role in CNI induced nephropathy. Polymorphism in the signal sequence genetically control the production of TGF-ß1 (16, 25, 26). Association between these TGF-ß1 gene polymorphisms and for instance progression of IgA nephropathy, reflux

nephropathy, atherosclerosis, hypertension, myocardial infarction, cardiomyopathy and accelerated graft vascular disease after HTx have been described (20, 27-32). We already reported the finding of an association between TGF- ß1 codon 10 (Leucine to Proline) gene polymorphism and CNI induced renal insufficiency in HTx recipients in 2000 (18). Now, with an extended follow up and a larger cohort, we found a highly significant association between TGF-ß gene polymorphisms and, the final consequence of CNI induced renal insufficiency, ESRF after heart transplantation. Pro carriers of either codon 10 or 25 had a 2.6 to 2.9 times increased risk for developing ESRF in univariable analysis. Also older age is an independent variable predicting ESRF as it is in the general population.

Survival of ESRF patients on renal replacement therapy was extremely poor with a median of only 1.5 years. This poor survival rate explains our underestimation of this very high prevalence of ESRF after heart transplantation: we actually see only 3 to 4 patients on renal replacement therapy at the same time.

In conclusion, after 21 years of cardiac allograft transplantation we found a high prevalence of ESRF and a highly significant influence of TGF-ß polymorphisms on CNI induced ESRF after heart transplantation in univariable analysis. In the multivariable analysis the influence of TGF- ß1 codon 10 gene polymorphism remained significant. The implications of our findings are that maintenance immunosuppressive regimens of cardiac allograft recipients with a TGF- ß1 codon 10 and probably also 25 gene polymorphism should no longer contain CNI, particularly because ESRF after heart transplantation results in extremely high mortality rate.

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Chapter 3

Patient survival after the diagnosis of cancer in renal transplant recipients: a nested case control study

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Abstract

Introduction
Malignancy is a well-known complication after renal transplantation. We studied the influence of cancer on patient survival in the Dutch renal transplant population in a nested case controlled analysis.

Methods
Between March 1966 and May 2008 15,227 renal transplantations in 12,805 recipients were registered in the Netherlands Organ Transplant Registry database. Total follow-up was 89,651 person years. We performed an analysis of patient and graft survival both from the day of transplantation and the diagnosis of cancer in recipients with invasive cancer. Recipients without invasive cancer, matched for gender, age and year of transplantation, served as a control group. For the survival analysis after the diagnosis of cancer, the matched control group consisted of patients with a functioning graft at the moment the index patient was diagnosed with cancer.

Results
Cancer had been registered in 908 (7.1%) patients, 630 (69%) of them died with functioning kidney, 510 (81%) due to their malignancy (at 8.2 years after transplantation, median). The median patient survival after transplantation was 11.9 versus 16.8 years in the study and control group respectively (p <0.001). The median patient and graft survival, after the diagnosis of cancer was 2.1 versus 8.3 (p<0.001) and 25 versus 22.4 (p<0.001) years in the study and control group respectively.

Conclusion
Mortality due to cancer is observed at a significantly later time after transplantation compared to mortality due to the other main lethal complications. It significantly affects life expectancy and carries a poor prognosis with a limited survival after diagnosis.

Introduction

The management of renal transplant recipients is changing over the years. Prevention of acute rejection has been our main concern in the early years, but with the development of better immunosuppressive medication, the increasing age of the transplanted population, and better patient and graft survival, the incidence of other complications increased in frequency. Currently, developing malignancies is a well-known and feared complication (1-7). The incidence of cancer increases over time and the relative risk of almost al tumor types are higher in the transplant recipients compared to the general population(8). Skin cancer is the most commonly observed type of malignancy with a reversed ratio of basal cell carcinoma (BCC) to squamous cell carcinoma (SCC) compared with the general population (2, 9-11). Studies in Dutch and Norwegian transplant recipients, a population with moderate sun exposure, showed that SCC occurred 65 to 250 times, BCC 10 times as frequently as in the general population (12-13) with a high risk of subsequent nonmelanoma skin cancer (14) . An analysis of 13,000 renal transplants performed in Australia and New Zealand showed a cumulative risk for developing at least one malignancy (excluding nonmelanoma skin cancer) of approximately 30% after 20 years (15).
Relative and absolute risk differ across patient groups and depending on patients' age, gender, primary cause of end stage kidney disease, race, prior malignancy, and graft survival. Better graft survival implies a higher cumulative dose of immunosuppression and thus a higher risk of cancer (5-6, 8). The use of immunosuppression and the possible limitation of treatment options in renal transplant recipients may have its influence on patient survival after the diagnosis of cancer (16). Studies describing patient survival after the diagnosis of cancer are scarce. Miao Y. et al performed a study for several common cancers, which showed that renal transplant recipients are diagnosed at a younger age and later stage of disease and have a significant worse outcome after the diagnosis of cancer compared with the general population (17). We studied the influence of developing cancer on patient survival in the Dutch renal transplant population. As a control group we did not use the general population but a nested case control group of renal transplant recipients with comparable other risk factors, in example cardiovascular disease and infection.

Materials and methods
Patients
After transplantation all renal transplant recipients were seen in the outpatient clinic of their transplant center. During every visit to their outpatient clinic, history was recorded and laboratory tests and urine analysis was performed. An electrocardiogram, chest X-ray and a cervical smear was performed once a year and on indication. On indication, more specific investigations were done (if there were abnormal findings in history, physical examination or other test results). For every transplanted organ, the

NOTR database form is completed yearly until the transplant fails or the patient dies. For this study, we retrospectively checked the medical records of the 15,227 renal transplantations in the NOTR database to identify recipients with a first post-transplant malignancy. Non-melanoma skin cancers and malignancies before transplantation were not included in this analysis. All malignancies were confirmed by pathological examination. We performed an analysis of patient survival from the day of transplantation in recipients developing their first post-transplant invasive cancer ('cases', non-melanoma skin cancer excluded). Subsequent malignancies in a different organ system, not considered as metastasis from the primary tumor, were counted separately. Recipients not diagnosed with invasive cancer, matched for gender, age and year of transplantation, served as a control group.

Furthermore we performed a survival analysis after the diagnosis of malignancy. In this analysis the control group, matched for gender, age and year of transplantation, consisted of patients with a functioning graft at the moment the index patient was diagnosed with cancer. The requirement for informed consent from patients was waived because the researchers received only anonymous data.

Immunosuppression

From 1966 until 1984 the standard immunosuppressive treatment for renal transplantation in the Dutch transplantation centers consisted of prednisolone (Pred) combined with azathioprine (AZA). Cyclosporine (CsA) was first registered in the NOTR as one of the immunosuppressive drugs after renal transplantation in 1979. From1984 the preferred immunosuppressive regimen for new renal transplant recipients was a combination of Pred and CsA, with or without AZA.

In 1992 mycophenolate mofetil (MMF) was first registered in the NOTR database as used in a renal transplant recipient as one of the immunosuppressive drugs. From 1996 onwards, the preferred immunosuppressive regimen for de novo renal transplant consisted of triple therapy with Pred, MMF and a calcineurine inhibitor, first CsA and later tacrolimus (Tac).

Induction therapy with anti-thymocyte globulin (ATG), OKT3 or anti-CD25 was not common practice, but only used as part of controlled clinical trials.

In case of acute rejection, patients were treated with methylprednisolone. If there was no response to methylprednisolone and/or in a T-cell mediated rejection with arteritis (type II rejection, Banff classification), ATG was given. Humoral rejection was treated with a combination of methylprednisolone, intravenous immunoglobulin's (Ivlg), plasmapheresis and Rituximab.

Statistical methods

Survival curves were made using the Kaplan-Meier method and the log-rank test was used to compare the survival rates. Data that did not follow a normal distribution are presented as medians and tested by Wilcoxon signed rank test.

The SSPS statistical package version 15.0 (®SPSS Benelux BV, an IBM Company) was used. *P*-values <0.05 were considered significant

Table 1:

Different types of cancer with patient survival censored for failure

Cancer site	Number of RTx with cancer	Median time from RTx to cancer Dx (years)	Number of patients died after cancer Dx	Patient survival rate (%)	Median patient survival after RTx (years)	Median patient survival after the Dx of cancer (months)
Primary brain tumors	11	4.5	6	45%	10.6	54
Tractus respiratorius	67	6.2	59	12%	8.1	5
Lip, oral cavity and pharynx	31	7.3	17	45%	14.3	69
Esophagus	15	16.5	9	40%	21	7
Stomach	13	10.2	12	8%	10.8	5
Colorectal	68	9.3	45	34%	14.7	15
Gall bladder & liver	18	9.7	16	11%	10.7	1
Pancreas	5	0.8	5	0%	0.8	2
Breast	48	6.5	21	56%	17	49
Gynecological tumor	35	5.4	14	60%	19	304
Prostate	41	3.8	11	73%	30	114
Kidney	29	7.3	12	59%	21.2	77
Urinary tract and bladder	48	3.6	21	56%	18.1	91
Melanoma	15	4.5	8	47%	15.3	39
Non-Hodgkin lymphoma	81	4.8	45	44%	11.6	26
Hodgkin lymphoma	10	7.0	3	70%	24	205
Leukemia	45	6.3	29	36%	9.6	10
Other malignancies	67	5.5	36	46%	13.7	24
Patients registered as died due to cancer of unknown primary site	261	unknown	261	0%	9.9	unknown
Total number	**908**		**630**	**31%**		

RTx=renal transplantation, Dx=diagnosis.

The median graft survival, censored for death, after the diagnosis of cancer was 25 (range 0-29) versus 22.4 (range 0-25) years in the cases versus the controls (p<0.001) (Figure 2).
The cause of death in patients diagnosed with cancer specified for the different types of cancer are shown in table 2.

Results

Between March 1966 and May 2008 15,227 renal transplantations, (12,060 deceased and 3167 living donor kidneys), in 12,805 recipients, (5163 female and 7642 male), were registered by the 7 Dutch transplantation centers in the Netherlands Organ Transplant Registry (NOTR) database of the Transplant Society (NTS). Total follow-up was 89,651 person years. The median age at transplantation of the total group was 44 (range 1-80) years. The median patient survival, censored for graft failure, was 18.9 (range 0-39) years. The median graft survival, censored for death, was 21.2 (range 0-39) years. The median overall uncensored graft survival was 9.3 (range 0-39) years.

Deceased patients and the causes of death:

In total 3173 patients died with a functioning graft. In 505 (16%) patients the cause of death was registered as due to an infection (at 3.5 years after RTx, median), in 161 (5%) patients a gastrointestinal complication (at 5.2 years after RTx, median), in 1117 (35%) patients cardiovascular disease (at 6.2 years after RTx, median) and 880 (28%) patients died of other causes. Malignancy was registered as cause of death in 510 (16%) patients (at 8.2 years after RTx, median).

Mortality due to cancer was observed at a significantly ($p<0.001$) later time after transplantation compared to mortality due to infection, gastrointestinal complications and cardiovascular disease.

Patients diagnosed with cancer:

At time of observation, a first post transplant malignancy was registered after 908 (7.1%) transplantations in 906 recipients. Of these 906 patients, 630 (70%) died with functioning kidney, 510 (56%) due to their malignancy. The median patient survival censored for failure after transplantation in these 906 renal transplant patients registered with a malignancy was 11.9 versus 16.8 years in renal transplant patients without malignancy ($p <0.001$). The results of patient survival for the different types of malignancies are shown in table 1.

Survival analysis after the diagnosis of cancer

The median patient survival, after the diagnosis of cancer was 2.1 (range 0-25) years in the study versus 8.3 (range 0-29) years in the control group without cancer ($p<0.001$) (Figure 1). In this last analysis the control group, matched for gender, age and year of transplantation, consisted of patients with a functioning graft at the moment the index patient (case) was diagnosed with cancer. The results of patient survival after the diagnosis of cancer, specified for the different types, are shown in table 1.

Table 2: Cause of death in renal transplant patients, with the diagnosesis of cancer.

Cancer site	Total number of patients	Total number of patients died	Cause of death				Other causes
			Cardio-vascular	Gastro-intestinal	Infection	Malignancy (% of dead patients who died due to their malignancy)	
Primary brain tumors	11	6 (55%)	0	0	0	4 (66%)	2
Tractus respiratorius	67	59 (88%)	4	0	1	45 (76%)	9
Lip, oral cavity and pharynx	31	17 (55%)	4	0	1	9 (53%)	3
Esophagus	15	9 (60%)	1	0	0	8 (89%)	0
Stomach	13	12 (92%)	2	0	0	10 (83%)	0
Colorectal	68	45 (66%)	3	4	2	28 (62%)	8
Gall bladder & liver	18	16 (89%)	0	0	1	14 (88%)	1
Pancreas	5	5 (100%)	0	0	0	5 (100%)	0
Breast	48	21 (44%)	2	0	1	13 (62%)	5
Gynecological tumor	35	14 (40%)	2	0	2	9 (64%)	1
Prostate	41	11 (27%)	3	0	2	4 (36%)	2
Kidney	29	12 (41%)	1	0	1	7 (58%)	3
Urinary tract and bladder	48	21 (44%)	2	0	2	12 (57%)	5
Melanoma	15	8 (53%)	1	0	0	5 (63%)	2
Non-Hodgkin Lymphoma	81	45 (56%)	2	2	8	29 (64%)	4
Hodgkin Lymphoma	10	3 (30%)	0	0	0	3 (100%)	0
Leukemia	45	29 (64%)	2	1	3	17 (59%)	6
Other malignancies	67	36 (54%)	2	3	1	27 (75%)	3
Patients registered as died due to cancer of unknown primary site	261	261 (100%)	0	0	0	261 (100%)	0
Patients without cancer	11,897	2543 (21%)	1086	151	480	0 (0%)	826
Total	**12,805**	**3173 (25%)**	**1117**	**161**	**505**	**510**	**880**

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Figure 1: Patient survival, censored for failure, after the diagnosis of cancer.
Patients with cancer (cases) versus the control group. The control group, matched
for gender, age and year of transplantation, consisted of patients with a functioning
graft at the moment the index patient was diagnosed with cancer.
Median patient survival, cases versus controls, was 2.1 (range 0-25) versus 8.3
(range 0-29) years, p<0.001.

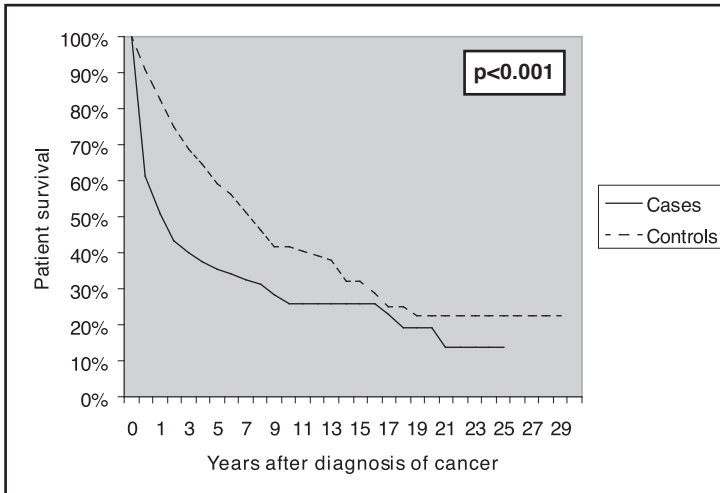**Figure 2:** Graft survival, censored for death, after the diagnosis of cancer.
Patients with cancer (cases) versus the control group. The control group, matched
for gender, age and year of transplantation, consisted of patients with a functioning
graft at the moment the index patient was diagnosed with cancer. Median graft
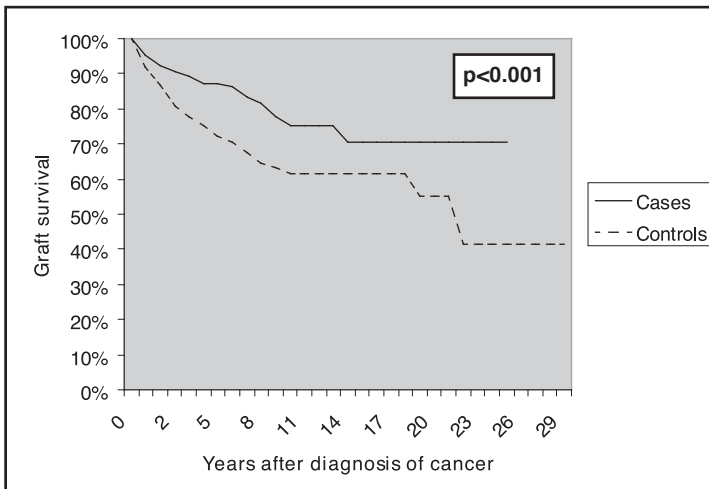survival, cases versus controls, was 25.0 (range 0-29) versus 22.4 (range 0-25)
years, p<0.001.

Discussion

Most studies in transplantation, describing patient survival after developing cancer, compare their results with the general population, showing a significant worse survival for the transplanted population. However, the vast majority of the general population is not at risk for the other known lethal side effects of immunosuppression, such as infection and cardiovascular disease. Our study shows that even compared with other renal transplant recipients, matched for gender, age, time after and year of transplantation, developing cancer after renal transplantation significantly affects life expectancy and carries a poor prognosis with a limited patient survival after the diagnosis. Moreover, the mean follow-up of our patients is shorter than the time to the diagnosis of cancer, which implicates that a higher number of patients will be diagnosed with cancer in future. Furthermore, we are not informed about the cancer risk after transplant failure, which also may contribute to patient survival in a negative way (18).

In patients with types of cancer with relative good prognosis, mortality tends to shift to other causes of death, (e.g. cardiovascular disease). For instance, nearly all patients who died and had a history of cancer of the esophagus (89%), stomach (83%) or pancreas (100%), died from their malignancy. In contrast, this proportion was smaller in patients with cancer of the prostate (36%), urinary tract and bladder (53%) (Table 2). However, in due time, patients surviving cancer have a comparable death risk and risk for graft failure as the control group, as can be deducted from the parallel slopes of the curves in figure 1 and 2, after apparently 8 years.

When patient survival is curved (Figure 1) the sharp early decrease in survival can easily be explained by the complications of cancer. Moreover, a certain reluctance to treat renal transplant recipients with nephrotoxic agents might lead to a further reduction in survival after the diagnosis of cancer. The significant difference between cases and controls in graft survival, censored for death, is probably the result of censoring.

We also noted that mortality due to cancer is observed at a significantly later time after transplantation compared to mortality due to the other main lethal complications. Nevertheless, cancer is the 2nd most frequent cause of death after renal transplantation. Besides genetic predisposition, race, age, environment, duration of uremia and chronic antigen stimulation, the higher risk of cancer after transplantation is partially due to an impaired immune surveillance, a higher incidence of viral infections and an impaired capability to repair DNA damage. The last three factors can be attributed to the chronic use and cumulative dose of immunosuppression. Early minimization of the immunosuppressive load could therefore be successful in the prevention of this late complication after kidney transplantation.

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Chapter 4

Reduction of immuno-suppressive load in renal transplant recipients with a low donor specific cytotoxic T-lymphocyte precursor frequency is safe

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Abstract

Background

Tapering of immunosuppressive medication is indicated to prevent long-term side effects. Recently, we have shown that renal transplant recipients can safely be converted from calcineurin inhibitors to MMF or AZA when their donor specific cytotoxic T lymphocyte precursor frequencies (CTLpf) are below $10/10^6$ PBMC. We wondered whether a low CTLpf also had predictive value when immunosuppressive medication was reduced in patients only on MMF or AZA and steroid medication.

Methods

Renal transplant recipients with stable renal function, at least two years after transplantation and with low ($<10/10^6$ PBMC) CTLpf were included. Their MMF or AZA dose was reduced to 75% at 4 months and to 50 % of the original dose at 8 months after inclusion. Endpoint of the study was 12 months after inclusion or developing acute rejection.

Results

Forty-five patients have reached the one-year follow up endpoint. Their median time after transplantation was 4.2 years (range 2.0-15.5 years). Acute rejection was seen in one patient only, who had discontinued all his medication.

Conclusion

In patients with low CTLpf long after kidney transplantation a 50% reduction of immunosuppression is safe and further decreasing their immunosuppressive load is the obvious next step.

Introduction

Despite the fact that early graft survival in renal transplant recipients has been markedly improved there are still a lot of problems to solve. The long-term outcome of renal transplantation is influenced by the occurrence of chronic allograft nephropathy and side effects of the immunosuppressive medication such as nephrotoxicity, cardiovascular disease and malignancies. Of all deaths after renal transplantation, 16 to 36% are the results of accelerated cardiovascular mortality and 9 to 12% of early malignancy (United States Renal Data System 1999). Evidently, tapering of the immunosuppressive load is indicated to prevent these side effects and to improve the long-term survival of these patients, provided that no rejection will occur. To reduce the risk of developing rejection, it is very important to select a patient group on immunological grounds in which tapering of immunosuppressive medication is safe. Finding a reliable immunological assay that makes it possible to make this selection is an important goal in organ transplantation.

Recently, we performed a study with renal transplant recipients who were at least one year after transplantation and had a stable renal function, in whom their immunosuppressive medication was converted from calcineurin inhibitors and prednisone to mycophenolate mofetil (MMF) or azathioprine (AZA) and prednisone. This conversion was safe, without developing acute rejection, if their donor specific cytotoxic T-lymphocyte precursor frequency (CTLpf) was low (<10 per million PBMC's) (1). We wondered whether further reduction of the immunosuppressive load in patients not on calcineurin inhibitors and with a low donor specific CTLpf was possible too.

Patients and methods

Forty five renal transplant recipients who were at least two years after transplantation with stable renal function, no proteinuria, without acute rejection in the last 6 months and with low (< 10 per million PBMC's) donor specific cytotoxic T-lymphocyte precursor frequencies (CTLpf) before reduction of their immunosuppressive medication were included in this prospective study. HLA identical transplantations were excluded. Donor spleen cells of a deceased donor or living donor PBMC had to be available to measure CTLpf. CTLpf was measured using a limiting dilution assay as described before (1). All patients had a high CTLpf against third party. At time of inclusion (T0) all patients were treated with AZA 2 mg/kg/day or MMF 2 gr/day in combination with prednisone 10 mg/day. The AZA or MMF dose was reduced to 75% at 4 months (T4) and to 50% of the original dose at 8 months after inclusion, reaching maintenance treatment of AZA 1 mg/kg/day or MMF 1 gr/day combined with prednisone 10 mg/day. Endpoints of the study were end of follow up at 12 months after inclusion (T12), death, graft loss or developing acute rejection. The diagnose of acute rejection was made on clinical grounds (rising creatinine, proteinuria, oliguria, fever and/or graft tenderness) and confirmed by core needle biopsy. Acute rejection was treated with methyl prednisolone 1 gram i.v. a day for 3 consecutive days.

Statistical analysis

Numerical data were compared using paired t-test or the Wilcoxon signed rank test. The results are reported as the mean with standard deviation or the median with range. A two-tailed P-value of <0.05 was considered to be significant.

Results

The baseline characteristics of the patients at time of inclusion are shown in table 1. Thirty-seven patients (82%) have reached T12. One patient (2%) developed acute rejection after the first dose reduction (T4), but he confessed later on that he discontinued his assigned medication completely. He was treated as described before with one course of prednisolone. His creatinine level stabilized after this treatment. Three patients (6%) refused to follow the study protocol after one dose reduction, because their fear to develop acute rejection. One patient (2%) developed biopsy proven chronic allograft nephropathy after the first dose reduction. One patient (2%) developed glaucoma. One patient has not yet reached the end of follow up at this moment. The mean creatinine level was 111 ± 36 µmol/l at T0 versus 117 ± 40 µmol/l at T12 (p=0.46) (figure 1). None of the remaining patients developed proteinuria during follow-up. The median proteinuria level was 0.10 g/l (0.10-0.25 g/l) at T0 versus 0.10 g/l (0.10-0.23 g/l) at T12 (p=0.51) (figure 2).

Table 1. Baseline characteristics of the patients at time of inclusion.

Sex (female/male)	14/31
Age (years)	48 ± 12.5
Median time after transplantation (years)	4.3 (range 2.0-15.5)
Living/deceased donor	33/12
MMF/AZA at inclusion (number of patients)	26/16
Serum creatinine (*µ*mol/l)	103 (59-243)

Conclusion

Trying to prevent long-term side effects by tapering the immunosuppressive load must be weighed against the risk of developing rejection and/or graft loss. Testing for T-cell reactivity to select a patient group in which tapering of medication is safe is suggested by many groups (2-8). Recently, we have shown that conversion of immunosuppressive medication in renal transplant recipients is safe when their donor specific CTLpf is low (1). In the present study, we have shown that a fifty percent reduction of the MMF or AZA dose in renal transplant recipients with low donor specific CTLpf is a safe procedure. There was a small, but clinical non-relevant increase of serum creatinine levels reflecting the normal loss of glomerular filtration rate within one year. No acute rejection occurred in the patients who followed the study protocol. Further reduction of the immunosuppressive load in this patient group and compare them with a patient group with high donor specific CTLpf is the obvious next step.
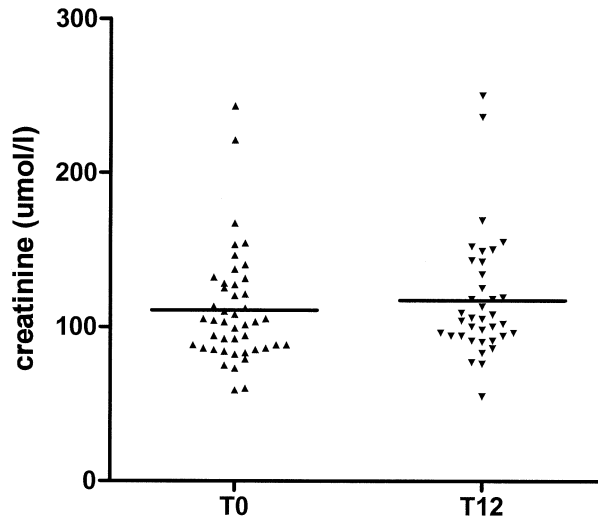
Figure 1: Serum creatinine levels at time of inclusion (T0) and at the end of follow-up (T12).
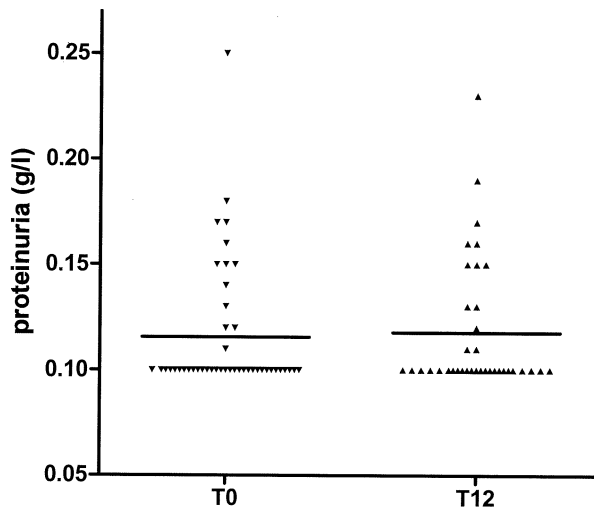

Figure 2: Proteinuria at time of inclusion (T0) and at the end of follow-up (T12).

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Chapter 5

After discontinuation of calcineurin inhibitors, tapering of mycophenolate mofetil further impairs donor-directed cytotoxicity

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Abstract

Background
Recently, we described a significant decrease in donor-specific cytotoxic T-lymphocyte precursor frequency (CTLpf) after discontinuation of CNI, while the proliferative capacity in mixed lymphocyte culture (MLC), and the number of IFN-γ producing cells (pc) in Elispot remained unchanged.

Methods
We tested T-cell reactivity in calcineurin inhibitors (CNI) free patients with stable renal graft function, on MMF or AZA plus prednisone, who were tapered to 50% of their MMF or AZA dose.

Results
Furthermore, tapering of the MMF or AZA dose resulted in a decrease of donor-reactive CTLpf in all patients with detectable CTLpf. Detectable numbers decreased from a median of 32 to 8 CTLp per 10^6 PBMC. No effect on third-party reactive CTLpf was found, while the T-cell reactivity to donor and third-party cells as tested in MLC and in IFN-γ Elispot was not affected either by tapering of immunosuppression. Third-party reactivity was significantly higher than donor-specific reactivity in all tests. A control group showed no changes in any of the in vitro assays.

Conclusion
Both withdrawal of CNI and tapering of MMF or AZA dose decreases the donor-specific CTLpf. Our data suggest that reduction of immunosuppression results in a specific decrease of donor-directed cytotoxic capacity of immunocompetent cells, while their proliferation and cytokine production capacity remained unchanged. Immunosuppression hinders development of cytotoxic nonresponsiveness.

Introduction

Advances in immunosuppression spectacularly improved the success of organ transplantation. Nowadays, over-immunosuppression has emerged as the major problem after transplantation (1). Therefore, the focus of immunosuppression management has shifted from the prevention of acute rejection to minimization of the total immunosuppressive load.

In earlier studies, it was demonstrated that elimination of calcineurin inhibitors (CNI: cyclosporine A and tacrolimus) in patients treated with mycophenolate mofetil (MMF) and prednisone at 3 or 6 months after transplantation (2, 3) or in patients converted to MMF or azathioprine (AZA) (4) resulted in an improvement of renal function and hypertension. In these studies, CNI were withdrawn within 1 year after transplantation, and the incidence of acute rejection was between 15 and 25%. Recently, we performed a study in which CNI were withdrawn in 51 renal transplant recipients who were at least 2 years after transplantation. Thereafter, patients received only MMF or AZA in combination with prednisone. Only one reversible acute rejection occurred (5). In that study we observed a significant decrease in the number of donor-specific cytotoxic T-lymphocyte precursors (CTLp) after CNI withdrawal, while no difference was found in third-party reactive CTLp frequency (CTLpf), donor and third-party mixed lymphocyte culture (MLC), and the number of IFN-γ pc directed to donor or third-party cells.

Apart from the side-effects of immunosuppression per se, e.g. infections and malignancies, MMF and AZA also have their own agent-specific side-effects, like anaemia, leukopenia, gastrointestinal and liver problems (1, 6). As we had observed no increase in T-cell reactivity after discontinuation of CNI in our patients, the immunosuppression was reduced further to approximately 50% of the MMF or AZA dose in an attempt to reduce the risk for side-effects (7).

In the present study, we questioned whether 50% reduction of the MMF or AZA dose has an effect on immune reactivity in CNI-free patients with stable renal function more than 2 years after transplantation, on MMF or AZA plus prednisone. T-cell reactivity of patients' PBMC against donor and third-party cells was tested in limiting dilution assay to determine the number of CTLp, in MLC to determine the proliferative capacity, and in Elispot-assay to determine the number of IFN-γ producing cells. The results of the assays were compared before and after tapering immunosuppression, and with a control group in which the MMF and AZA dose remained unchanged.

Patients and Methods

Patients

Recently, we described a prospective study, in which CNI were withdrawn more than 2 years after kidney transplantation (5). Thereafter the patients received MMF or AZA plus corticosteroids. After informed consent, a number of patients agreed to a further dose reduction of MMF or AZA to approximately 50%.

All patients were recipients of a first kidney graft, had stable graft function without proteinuria (<0.5 g/L), and were free from rejection for at least 1 year. The patient characteristics are presented in Table 1.

At inclusion, patients (n=19) received a median dose of 2000 mg/day MMF (n=13: range: 1000 - 2000) or 150 mg/day AZA (n=6: range: 100 - 175). The MMF or AZA dose was tapered in two steps of 4 months to a median dose of 1000 mg/day MMF (range: 500 - 1000) or 62.5 mg/day AZA (range: 50 - 75) (Table 1). The T-cell reactivity was analysed at inclusion and at 4 months after the last dose reduction (one year after inclusion). A group of 8 patients (MMF: n=5, AZA: n=3), not tapered in immunosuppression, served as control. T-cell reactivity was analysed at time of inclusion and one year later.

Table 1:

Patients characteristics of kidney transplant recipients who were tapered in their MMF or AZA dose.

Patient	LR[*] or PM[#]	Time after KTx[^] (in years)	Immunosuppression before tapering		Immunosuppression after tapering	
			MMF or AZA (mg/d)	Pred (mg/d)	MMF or AZA (mg/d)	Pred (mg/d)
1	LR	6.92	150 AZA	10	50 AZA	10
2	LR	3.43	2000 MMF	7.5	1000 MMF	7.5
3	PM	3.56	1000 MMF	7.5	500 MMF	7.5
4	LR	6.62	175 AZA	10	75 AZA	10
5	LR	3.16	2000 MMF	10	1000 MMF	10
6	PM	2.44	2000 MMF	10	1000 MMF	10
7	PM	5.49	100 AZA	10	50 AZA	10
8	PM	3.65	2000 MMF	10	1000 MMF	10
9	PM	4.15	2000 MMF	10	1000 MMF	10
10	LR	5.91	2000 MMF	10	1000 MMF	10
11	PM	3.67	2000 MMF	10	1000 MMF	10
12	LR	4.28	150 AZA	10	75 AZA	10
13	PM	2.56	2000 MMF	7.5	1000 MMF	7.5
14	LR	2.92	2000 MMF	10	1000 MMF	10
15	LR	4.22	2000 MMF	10	1000 MMF	5
16	LR	2.01	2000 MMF	10	1000 MMF	10
17	LR	2.86	2000 MMF	10	1000 MMF	10
18	LR	2.94	150 AZA	10	75 AZA	10
19	LR	11.19	100 AZA	10	50 AZA	10

* LR : kidney donation from a living-related donor
# PM: kidney donation from a deceased donor
^ KTx: kidney transplantation

PBMC and spleen cell sampling

Heparinised blood samples (35 ml) from patients and living-related donors were drawn. PBMC were isolated from heparinised blood as described before (8) and stored at -140ºC until use. Spleen cells were obtained by mechanical dissociation of small pieces of spleen derived from the organ donor (8). Subsequently, the cell suspension was filtrated through a 70 μm cell strainer (BD Falcon, Bedford, MA) and washed. Thereafter, the cells were centrifuged over a Ficoll-Paque (Amersham Biosciences, Uppsala, Sweden) density gradient, collected, washed and stored at -140°c. MLC and tetanus toxoid (TET) stimulation

MLC were set up as described earlier (5) with 100 μl of a 5×10^4 responder PBMC in triplicate wells and 100 μl of (a) 5×10^4 irradiated (40 Gy) PBMC or spleen cells from the donor; (b) 5×10^4 irradiated (40 Gy) PBMC from a third-party; (c) tetanus toxoid (TET: RIVM, Bilthoven, The Netherlands) at 7.5 lf/well final concentration as nominal antigen to test the memory immune response; (d) PHA (2 μg/ml of purified PHA (Murex Biotech Ltd) to check the viability of the cells; and (e) culture medium. After 7 days (for PHA 3 days), proliferation was measured by incorporation of 3H-thymidine added during the last 8 hours of culture. The stimulation index (SI) was calculated by the ratio of the cpm obtained in the presence of antigen to the cpm in the absence of antigen.

Only the results of viable cells (SI ^{3} 50) from patient, donor and third-party were analyzed in the described results. For some patients not enough cells were available to perform all tests.

Elispot assay

One hundred ml containing 10^5 patient PBMC in complete culture medium was added to 100 ml 10^5 irradiated (40 Gy) PBMC or spleen cells derived from the donor or third-party in round-bottomed wells (6-fold) of a 96-well plate (Nunc, Roskilde, Denmark) as described earlier (9). To control the influence of irradiation on cytokine production, PBMC were incubated with irradiated PBMC of the same patient (autologous control). We also stimulated the cells with TET as recall antigen and PHA as positive control. After 40 hours of incubation the non-adherent cells were harvested and transferred in triplicate to a flat-bottom 96-well Elispot plate (U-CyTech B.V. Diagnostics, Utrecht, The Netherlands) pre-coated with a mouse anti-human IFN-γ monoclonal Ab and post-coated with PBS containing 1% BSA. Cells were incubated in the Elispot plate for 5 hours to allow spot formation. The spots were counted automatically by using a Bioreader 3000 Elispot-reader (BioSys GmbH, Karben, Germany).

Limiting dilution assay (LDA)

Limiting dilution cultures were set up as described previously (5). In brief, 24 replicates of graded number responder PBMC were titrated in 7-step double dilutions starting from $5x10^4$ to 781 PBMC/well and stimulated with irradiated (40 Gy) donor or third-party PBMC/spleen cells ($5x10^4$ cells/well) in 200 µl culture medium [RPMI-1640-DM (GibcoBRL, Scotland, United Kingdom) supplemented with 100 IU/ml of penicillin (Cambrex, Verviers, Belgium) and 100 mg/ml of streptomycin (Cambrex) and 10% pooled heat-inactivated and filtered (0.20 µm sterile syringe filter, Corning Incorporated, Corning, NY, USA) human serum, that was tested for adequate cell growth support in mixed lymphocyte cultures] containing recombinant IL-2 (200 U/ml, 12.2 ng/ml IL-2; proleukin: Chiron BV, Amsterdam, The Netherlands). Additionally, 24 wells contained stimulator cells alone. After 7 days of culture, each well was individually tested for cytolytic activity against $5x10^3$ Europium-DTPA labelled target cells [T-cell blasts, cultured with PHA (Murex Biotech Ltd, Kent, England) and rIL-2 (Chiron)]. After 4 hours of incubation, the plates were centrifuged and 20 µl of the supernatant was harvested. Fluorescence of the released Europium was measured and was expressed in counts per second (cps). The mean cps of the wells in which only stimulator cells were present, were considered as background. Experimental wells were scored positive, if the counts in that well exceeded the mean + 3 x SD of the wells in which only stimulator cells were present. For each cell concentration the number of negative wells was determined and used to calculate the frequency with a computer program designed by Strijbosch et al. (10). The CTLpf was expressed as the number of CTL per 10^6 PBMC.

Statistical analysis

The significance of differences between the tests before and after tapering immunosuppression was analyzed using the paired Wilcoxon signed rank test. Data concerning the presence of detectable donor-specific CTLpf of the patients before or after tapering were analyzed with Fischer's Exact Test.

Results

Clinical results

After tapering the immunosuppressive dose to 50% of the original MMF or AZA dose the serum creatinine level remained unchanged [100%: median 103 µmol/l (range: 59-175) vs. 50%: 113 µmol/l (range: 55-182)], and was comparable with the control group [T=0 months: 119 µmol/l (range: 79-156) vs. T=12 months 115 µmol/l (range: 65-182)]. One year after dose reduction the serum creatinine levels remained still unchanged (median: 107 µmol/l (range: 53-174). None of the patients developed rejection or proteinuria (>0.5 g/l).

The MPA levels decreased significantly from a median of 2.42 mg/l (range: 1.54-8.22) to 1.40 (0.50-3.07) after tapering the MMF dose (p=0.0002).

From all patients (tapered n=19, controls n=8) we isolated PBMC before and after dose reduction of the immunosuppressive load to perform in each sample the T-cell reactivity against donor and third-party reactivity in MLC, Elispot, and limiting dilution assay to determine the number of CTLp. In addition, TET-reactivity was used to test the general immune response. Only data are presented of viable cells (SI ^{3} 50) from patient, donor and third-party. For some patients not enough cells were available to perform all tests.

Mixed lymphocyte culture (MLC)

The donor-specific MLC did not change as a consequence of tapering the immuno-suppressive load [100% immunosuppression (T=0): median SI 13 (range: 1-570) vs. 50% immunosuppression (T=12) SI 11 (1-559)]. Also in the control group, the donor-specific MLC remains unchanged [T=0 months: SI 2 (range: 1-4) vs. T=12 months SI 3 (1-35)].

Third-party reactivity in both tapered and control group was significantly higher than the donor-specific MLC (p=0.001 vs. p=0.008, respectively).

The third-party specific MLC was not different before and after tapering immunosup-pression [T=0 months: median SI 107 (range: 14-685) vs. T=12 months: SI 157 (25-846)]. The third-party MLC did not change in time in the control group [T=0 months: SI 152 (range: 62-185) vs. T=12 months: SI 112 (23-204)].
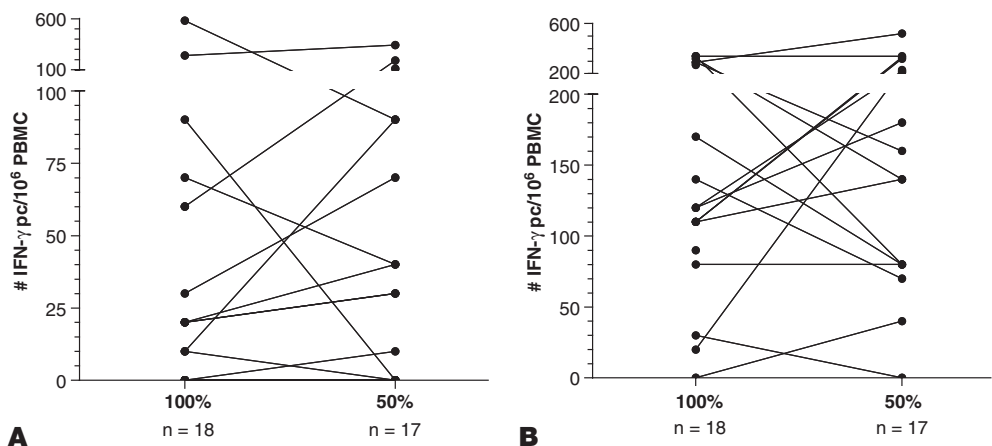

Figure 1
Number of IFN-γ producing cells directed to donor cells (A) or to third-party cells (B) in PBMC from patients before (100%) and after (50%) tapering the immunosuppressive dose to 50% of the original dose.

Frequency of IFN-γ producing cells (pc)

Neither the number of IFN-γ pc directed to donor antigens (Figure 1A) nor directed to third-party antigens (Figure 1B) was different in the period before and after tapering immunosuppression. The response to third-party cells was significantly higher than to donor antigens (p<0.0001).

In the control group, no difference in time was found in donor [T=0 months: 40 IFN-γ pc/10^6 PBMC (range: 0-170) vs. T=12 months: 40 IFN-γ pc/10^6 PBMC (0-150)] and third-party [T=0 months: 140 IFN-γ pc/10^6 PBMC (range: 0-270) vs. T=12 months: 80 IFN-γ pc/10^6 PBMC (0-450)] reactivity.

Tetanus toxoid (TET) reactivity

The reactivity to TET measured in a proliferation assay did not change after tapering immunosuppression [before tapering: median SI 4 (range: 1-142) vs. after tapering: SI 4 (1-270)]. The TET reactivity in the control group remained stable in time [T=0 months: SI 3 (1-22) vs. T=12 months: 12 (1-559)].

Also the number of IFN-γ producing cells specific for TET was comparable before and after tapering immunosuppression [before: 40 IFN-γ pc/10^6 (0-620) vs. after: 15 IFN-γ pc/106 (0-395)], and in the control group [T=0 months, 25 IFN-γ pc/10^6 (0-255) vs. T=12 months, 78 IFN-γ pc/10^6 (0-595)].

Cytotoxic T-lymphocyte precursor frequency (CTLpf)

Before tapering the immunosuppressive load, the donor-specific CTLpf was detectable (≥10/106 PBMC) in 44% (7/16) of the patients tested in CTLpf (Figure 2A). In the control group 43% (3/7) of the patients had detectable donor-specific CTLpf. The donor-specific CTLpf of the 7 patients with detectable CTLpf (n=6 MMF, n=1 AZA)
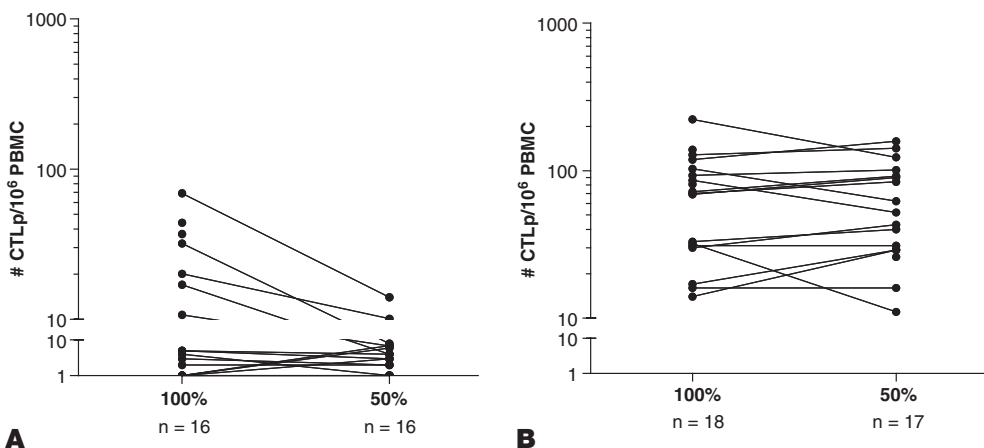

Figure 2
CTLpf determined against donor cells (A) or against third-party cells (B) in patients before (100%) and after (50%) tapering the immunosuppressive dose to 50% of the original dose.

decreased in all cases after tapering the immunosuppressive load from median of $32/10^6$ PBMC (range: 10-69) to $8/10^6$ PBMC (range: 4-14). This pattern was not seen in the control group. Third-party reactivity in both the tapered and control group was significantly higher than the donor-specific CTLpf (p<0.0001), but tapering immunosuppression had no effect on third-party CTLpf (Figure 2B).

Discussion

After clinical transplantation life-long immunosuppression is deemed necessary. As minimizing immunosuppression is presumed to be beneficial, avoiding or tapering of one of the immunosuppressive drugs is recommended (1, 11). In our transplant center, all patients with stable kidney function and 2 years after transplantation were withdrawn from CNI to receive MMF or AZA in combination with low dosed Pred (5). Thereafter, the MMF and AZA dose were tapered to half of the original dose (7).
In the present study, we investigated T-cell reactivity during tapering the MMF or AZA dose. Comparable with our results after withdrawal of CNI (5), we found a decrease in donor-reactive CTLpf and no change in third-party reactive CTLpf, donor and third-party MLC, frequency of IFN-γ pc directed to donor or third-party antigens, and TET reactivity after tapering the immunosuppression. After tapering the immunosuppressive dose, the hyporesponsiveness of donor-specific CTLp became even more evident. Although, our data have to be confirmed in a larger group of transplant patients who will be reduced in their immunosuppressive load versus those who are not, we suggest that a specific down-regulation of CTLp directed to donor antigens presented by the direct presentation pathways occurred during tapering the MMF or AZA dose. This downregulation might be due to anergy, deletion or regulation. Because IL-2 reverses the anergic state of cells (12), and we added exogenous IL-2 to the LDA cultures to determine CTLpf, anergy of donor-specific CTLp is an unlikely cause of the low numbers of donor-specific CTLp.
Mycophenolic acid (MPA) was shown to increase apoptosis of human T-cell lines (13) and deletion of *in vivo* activated T-cells (14). If deletion of donor-activated CTLp occurs during MMF treatment, we expect that after tapering the MMF dose an increase in number of CTLp should be detected. In contrast, we found a decrease in CTLpf. Therefore, we assume that suppression of regulatory T-cells (Treg) by MMF or AZA is a more convenient explanation of the low number of donor-specific CTL after tapering the MMF or AZA dose.
Regulatory T-cell suppressor function has been demonstrated to be dependent on the presence of IL-2 (15, 16). Recently, IL-2 signalling has been shown to be necessary for the survival of FoxP3+ Treg in vivo (17). Before, we have shown that CNI discontinuation led to increased numbers of Treg (5), probably as the result of the reducing effect of CNI on IL-2 production. We also have shown that CNI reduces FoxP3 expression in human cells (18). In mice it was demonstrated that exogenous IL-2 can overcome reduced FoxP3 expression and the functional defect in Treg induced by CsA (19).

Less is known about the relevance of MMF and AZA on Treg function. The antipro-liferative agents MMF and AZA prevent expansion of alloactivated T and B-cells. Recently, it was shown in a mice model that MMF and AZA showed opposite effect on induction of regulatory cells after intratracheal delivery of donor splenocytes. AZA abrogated such induction, whereas MMF could promote the generation of Treg (20). In contrast to CNI, MMF did result in reduced FoxP3 gene expression when murine Tregs were exposed in vitro to immununosuppressive medication, while MMF inter-fered with Treg related protection in an acute graft-versus-host disease mice model (19). Thus, MMF could have an effect on Tregs *in vivo*.

The nature of dendritic cells (DC) has a major impact on the ability to induce Treg. DC mediated induction of Tregs includes, next to cell-cell contact and IL-2, CD80/CD86 interactions (21). Preferential expansion of FoxP3+ T-cells was found in the presence of mature DC compared to immature DC (21). The active metabolite of MMF, MPA, affects the phenotype and function of DC by reducing the expression of costimulatory molecules (22). Therefore, the suppressive effect of Treg exposed to MPA could be hindered by an indirect effect of MPA on DC. This may explain that after reducing the MMF dose, the donor-specifc CTLpf decreases, because of higher Treg activity.

Waldmann et al. (23) reported that mechanisms ranging from anergy and deletion to regulation should not be seen as distinctive mechanisms, but these may all interact with each other. This interaction is also suggested by a recent study of our group. Tregs of patients with positive MLC to donor-antigens could prevent rejection and allow stable graft function, while in other patients donor responder cells are no longer present in periphery, and therefore there are no cells to suppress (24).

In summary, we conclude that in kidney transplant recipients reduction of immuno-suppressive load leads to lower CTLp activity.

Acknowledgement

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Chapter 6

Discontinuation of calcineurine inhibitors treatment allows the development of FOXP3+ regulatory T-cells in patients after kidney transplantation

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Abstract

This study investigated specific gene expression profiles in patients with donor-specific cytotoxic-hyporesponsiveness, reflected by cytotoxic T-lymphocyte precursors frequency (CTLp). The effect of calcineurin inhibitor (CNI) withdrawal was studied on markers for cytotoxicity(perforin, granzyme B), apoptosis(Fas,FasL), Th1 and Th2 cytokines(IL-2, IL-10), Th1 and Th2 transcription factors(T-bet, GATA 3), Th17 transcription factor and cytokine(RORγt, IL-17), and for immune regulation/activation(CD25, FOXP3). Peripheral blood samples from renal allograft recipients (n=18), more than 2 years after transplantation with stable renal function were analyzed before and 4 months after CNI withdrawal. Additionally, systolic and diastolic blood pressure, cholesterol, serum creatinine and proteinuria were evaluated and no significant differences were measured before and after CNI withdrawal. However, CNIs' discontinuation influenced peripheral gene expression profiles. After CNI withdrawal the mRNA expression of Granzyme-B, Perforin, Fas, FasL, T-bet, GATA3, CD25 were significantly lower than during CNI treatment. After CNI discontinuation, donor specific CTLp frequency decreased, while FOXP3 expression discriminated between detectable and non-detectable donor-specific cytolysis reactivity; FOXP3 transcript values were highest in absence of donor-specific cytotoxicity (p<0.01). Our study shows, CNI withdrawal in stable kidney transplant recipients 2 years after transplantation, is safe. Moreover, discontinuation of CNIs' treatment allows FOXP3+ regulatory T-cells development, resulting in a significant decrease of anti-donor immune reactivity.

Introduction

Over the past years there has been a remarkable decline in acute rejection and early graft loss rates after kidney transplantation [1, 2]. Unfortunately, this positive outcome is accompanied by specific side effects such as nephrotoxicity, hypertension, lipid disorders, diabetes mellitus and an increased incidence of infections and malignancies [3]. Therefore, dose reduction or withdrawal of CNI (e.g., cyclosporine, tacrolimus) is advisable, provided that no rejection or graft loss will occur. Previous studies, in which CNI were replaced by mycophenolate mofetil (MMF) or azathioprine (AZA), reported improved renal function with low rates of hypertension [4] though a relative high acute rejection rate did occur (15-25%) [5-7]. However, these studies were performed within the first year after transplantation. Whether CNI withdrawal at later time points after transplantation also is associated with acute rejection and reduced CNI related side effects is still unknown. To assess at what time after transplantation CNI can be safely stopped, the use of a proper biomarker that measures anti-donor reactivity is helpful [8, 9]. For instance, we have shown that conversion from CNI to MMF or AZA is a safe procedure when their donor-specific cytotoxic T lymphocyte precursor frequency (CTLpf) is low [9].

Nowadays, it is evident from *in vitro* and animal studies that immunosuppressive agents interfere in both immune activation cascades as in the immunosuppressive counter mechanisms. For instance, CNI inhibit the gene transcription of IL-2, a cytokine with immunostimulatory as well as essential immunosuppressive actions [10]. IL-2 downregulates immune responses by both its pro-apoptotic actions and directs the function of CD4+CD25+CD127$^{-/low}$FOXP3+ suppressor T-cells [11].

In the present study, the effects of CNI withdrawal in patients > 2 year after kidney transplantation on both clinical and immunological parameters were studied. Parameters for kidney function, blood pressure and cholesterol were studied in concert with the biomarkers: CTLp frequencies, markers for cytotoxicity (perforin, granzyme B), apoptosis (Fas, FasL), Th1 and Th2 transcription factors and cytokines (T-bet, IL-2, GATA-3, IL-10), Th17 cells (RORγt, IL-17), and immune regulation (FOXP3).

Material and Methods

Patients

Stable renal transplant recipients (N=18) who were at least 2 years after transplantation (range 28-69 months) and rejection free in the last 6 months were withdrawn from the CNI tacrolimus or cyclosporine. At time of inclusion the patients were treated with tacrolimus or cyclosporine and AZA 2 mg/kg/day or MMF 2 g/day combined with prednisone 10 mg/day. Four weeks after inclusion, the patients were CNI free and used 2 g MMF (N=17) or 2 mg/kg AZA (N=1) combined with 10 mg prednisone a day (figure 1) [12]. Patients were removed from the study after developing graft loss or acute rejection. HLA identical living related transplantations were excluded.

The immune parameters CTLp frequencies, and mRNA expression levels of perforin, granzyme B, Fas, FasL, T-bet, IL-2, GATA-3, IL-10, RORγt, IL-17, and FOXP3 were measured before and 4 months after CNI withdrawal. From these patients, in the following part of our study, kidney function, blood pressure and cholesterol were studied before and after CNI withdrawal at 4 months. In addition, these clinical parameters were analyzed in the CNI free patients after further MMF dosage reduction (2 times 25%) at 24 months (figure 1) [12].
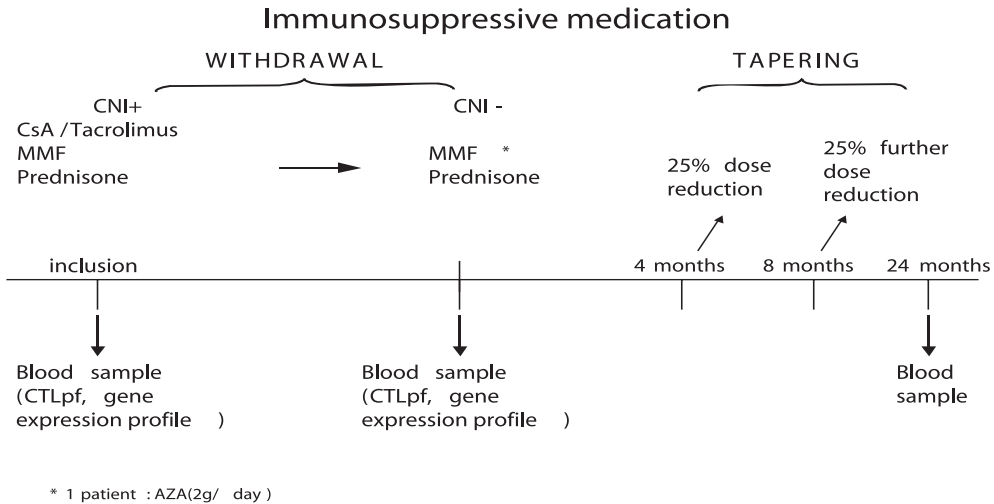

FIGURE 1. Study design. At the time of the conversion, the CNI medication was withdrawn. Blood samples were taken from 18 patients. Further tapering was made at 4 and at 8 months after withdrawal. At 24 months after CNI withdrawal blood samples were taken from patients for the needs of the clinical evaluation.

CTL precursors frequency

To determine the number of donor-specific cytotoxic T-lymphocytes, donor spleen cells in case of a deceased donor or living donor PBMC had to be available. Peripheral blood mononuclear cell (PBMC) samples of patients and living unrelated donors were isolated as described before, and stored at -140°c until use.

Cytotoxic T-lymphocyte precursor frequency (CTLpf) was measured using a limiting dilution assay as described previously [8] In brief, 24 replicates of graded number responder PBMC were titrated in 7-step double dilutions starting from $5x10^4$ to 781 PBMC/well and stimulated with irradiated (40Gy) donor or third party PBMC/spleen cells ($5x10^4$ cells/well) in 200 µl culture medium containing recombinant IL-2 (20 U/well, 12.2 ng/ml IL-2; proleukin: Chiron BV, Amsterdam, The Netherlands). Additionally, 24 wells contained stimulator cells alone. After 7 days of culture, each well was individually tested for cytolysis activity against $5x10^3$ Europium-DTPA labeled target cells (T-cell blasts, cultured with PHA and recombinant IL-2 (Chiron). After 4 hours of incubation, the plates were centrifuged and 20 µl of the supernatant was harvested.

Fluorescence of the released Europium was measured in a time-resolved fluorometer and was expressed in counts per second (cps). The mean cps of the wells in which only stimulator cells were present, were considered as background. Experimental wells were scored positive, if the counts in that well exceeded the mean three times the standard deviation of the wells in which only stimulator cells were present. For each cell concentration the number of negative wells was determined and used to calculate the frequency with computer program designed by Strijbosch et al [13] The CTLpf was expressed as the number per 10^6 PBMC.

Quantitative Real Time-PCR

Messenger RNA extraction from the PBMC, cDNA transcription and amplification was performed as described before [10]. In brief, total RNA was isolated using the High Pure RNA Isolation kit (Roche Applied Science, Penzberg, Germany), according to the manufacturer's instructions. RNA concentrations were measured using the RiboGreen® RNA Quantification Reagent And Kit (Molecular Probes, Eugene, OR,), and cDNA was synthesized from 500 ng RNA with random primers.

For the quantitative real time PCR analysis, the TaqMan technology (7700 Sequence Detector; Applied Biosystems, Foster City, CA) was applied according to the manufacturer's instructions. Messenger RNA expression levels of Granzyme B, Perforin, Fas, FasL, T-bet, IL-2, GATA3, IL-10, RORγt, IL17, FOXP3 and CD25 were quantified. The choice of primer and probe (IL-2, Granzyme B, Perforin, T-bet, GATA3) was defined using the primer express software (Applied Biosystems) and are listed in Table 1. FOXP3 (Hs00203958_m1) IL-10 (Hs00174086_m1) RORγt (Hs 00172858_m1) and IL-17A (Hs 00174383_m1) mRNA measurements were performed using Assay on Demand and pre-developed Taqman® PDAR assays to measure CD25 (4328847F), Fas (4318333F) and FasL (4319441F) concentrations (Applied Biosystems).

TABLE 1. Primer and probe sequences

Forward primer IL-2	5'-TTT-GAA-TGG-AAT-TAA-TTA-CAA-GAA-TCC-3'
Reverse primer IL-2	5'-TTC-TAG-ACA-CTG-AAG-CTG-TTT-CAG-TTC-3'
Probe IL-2	5'FAM-CCA-GGA-TGC-TCA-CAT-TTA-AGT-TTT-ACA-TGC-CC-TAMRA3'
Forward primer Perforin-1	5'-GTG-CCG-CTT-CTA-CAG-TTT-CCA-3'
Reverse primer Perforin-1	5'-CGT-AGT-TGG-AGA-TAA-GCC-TGA-GGT-A-3'
Probe Perforin-1	5'FAM-TGG-TAC-ACA-CTC-CCC-CGC-TGC-AC-TAMRA3'
Forward primer Granzyme-B	5'-CCC-TAC-ATG-GCT-TAT-CTT-ATG-ATC-TG-3'
Reverse primer Granzyme-B	5'-GAC-ATT-TAT-GGA-GCT-TCC-CCA-A-3'
Probe Granzyme-B	5'FAM-TGA-GCA-GCT-GTC-AGC-ACG-AAG-TCG-T-TAMRA3'
Forward primer T-bet	5'-AAT-GTG-ACC-CAG-ATG-ATT-GTG-CT-3'
Reverse primer T-bet	5'-TTC-AGC-TGA-GTA-ATC-TCG-GCA-TT-3'
Probe T-bet	5'FAM-TGG-TAG-GCA-GTC-ACG-GCA-ATG-AAC-TG-3'
Forward primer GATA-3	5'-CGG-TCC-AGC-ACA-GGC-AG-3'
Reverse primer GATA-3	5'-GGC-TGC-AGA-CAG-CCT-TCG-3'
Probe GATA-3	5'FAM-TGT-GTG-AAC-TGT-GGG-GCA-ACC-TCG-TAMRA3'

To quantify IL-2, Granzyme B, Perforin, T-bet and GATA3 transcript levels 5 ml cDNA was added to 20 µl PCR mixture containing 12,5 µl Universal PCR Master Mix (Applied Biosystems), 0,5 µl of sense primer (25 pmol), 0,5 µl anti-sense primer (25 pmol), 0,5 µl of FAM labeled probe (5 pmol) and 6 µl H_2O. To determine FOXP3, CD25, IL-10, Fas and FasL mRNA expression levels, we added 5 ml cDNA to 20 µl PCR mixture containing 12,5 µl Universal PCR Master Mix, 0.625 µl primer/probe mix and 6.875 µl H_2O. The PCR was performed after a first step of 2-min 50°C and 10-min 95°C by 40 cycles of 15 seconds at 95°C and 1 minute at 58°C (IL-2, Granzyme B, GATA3) or 59°C (Perforin, T-bet) or 60°c (FOXP3, CD25, Fas, FasL and IL-10). Each run contained several negative controls (no template), and two positive reference samples. For the quantification of mRNA expression levels we used the $2^{(40-Ct)}$ procedure as described by Bustin et al., and denoted target expression levels as copy number/500 ng RNA[14].

Statistical analysis

Statistical analysis was conducted using the Mann-Whitney test to compare the two groups, the one with the patients before the withdrawal of the CNI drug and the other, after the conversion. Results are reported as medians with range. A two-tailed P-value of <0.05 was considered to be significant.

Results

Clinical results

The clinical data are summarized in table 2a and table 2b and are in accordance with our previous published data [12]. After CNI discontinuation, and reduction of the immunosuppressive load 1 out of 18 patients developed proteinuria of 0,5 g/l, while the creatinine levels remained stable during time of observation (table 2). Furthermore, during the study period there were no significant differences between systolic and diastolic blood pressure (p_s =0.59 and p_d =0.13, table 2) and cholesterol levels (p=0.06).

TABLE 2a

	CNI+	CNI-	P value
Serum creatinine (µmol/l)	108.0 (61.0-238.0)	96.0 (59.0-221.0)	0.19
Proteinuria (g/l)	0.10 (0.10-0.17)	0.10 (0.10-0.54)	0.16
Systolic pressure (mmHg)	142 (125-200)	137 (120-180)	0.59
Diastolic pressure (mmHg)	80 (75.0-90.0)	80 (70.0-90.0)	0.13
Cholesterol (mmol/l)	6.10 (4.9-8.1)	6.00 (4.4-7.7)	0.06

TABLE 2b

	CNI+	CNI- (24 months)	P value
Serum creatinine (μmol/l)	108.0 (61.00-238.0)	106.0 (53.00-251.0)	0.71
Proteinuria (g/l)	0.10 (0.10-0.17)	0.10 (0.06-0.84)	0.55
Systolic pressure (mmHg)	142 (125-200)	140 (110-170)	0.34
Diastolic pressure (mmHg)	80 (75-90)	80 (65-90)	0.41
Cholesterol (mmol/l)	6.10 (4.9-8.1)	6.00 (4.2-7.2)	0.35

TABLES 2a and 2b. Clinical data as medians with a range, before, during, and two years following the conversion. Patients have been submitted to a further tapering of the new medication's dose at the 4th and 8th month after the medication's switch.

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CTLp frequencies before and after withdrawal of CNI

CTLp frequencies (CTLpf) were studied before CNI withdrawal and at 4 months after its discontinuation. During CNI treatment, PBMC samples from 13/18 patients were available, and after CNI discontinuation from all patients samples were present for CTLpf analysis. At inclusion 8/13 patients had detectable numbers of donor specific CTL ($\geq$10/10^6 PBMC) and after stopping CNI treatment donor specific CTLs were measurable in the peripheral blood of 5/13 patients. The donor specific CTL numbers decreased after withdrawal of CNI in each patient. The median CTLpf of all patients at the time of the inclusion was 19/10^6 (range 0-548/10^6 PBMC) and after CNI withdrawal 7/10^6 (range 0-208/10^6 PBMC, p=0.03, figure 2A). All patients had detectable CTLpf against third-party antigens before CNI discontinuation (figure 2B).
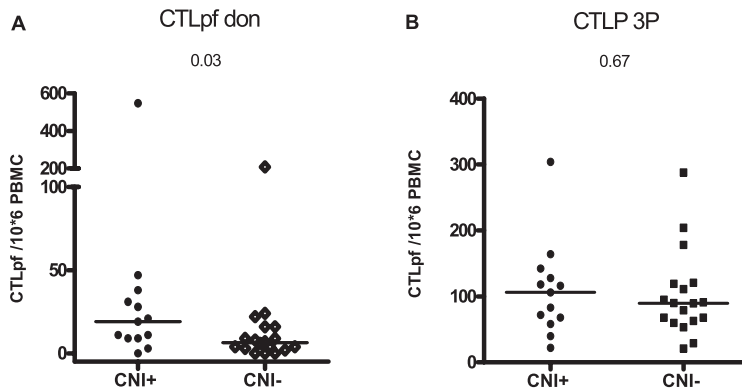
FIGURE 2 A and B. Analysis of cytotoxic T-lymphocyte precursor frequency (CTLpf/10^6 PBMC) against donor-specific and HLA 2-2-2 mismatched third-party cells, respectively, before (CNI +) and 4 months after (CNI -) withdrawal of CNI. The statistical difference, the median and the range between the groups are also evidenced.
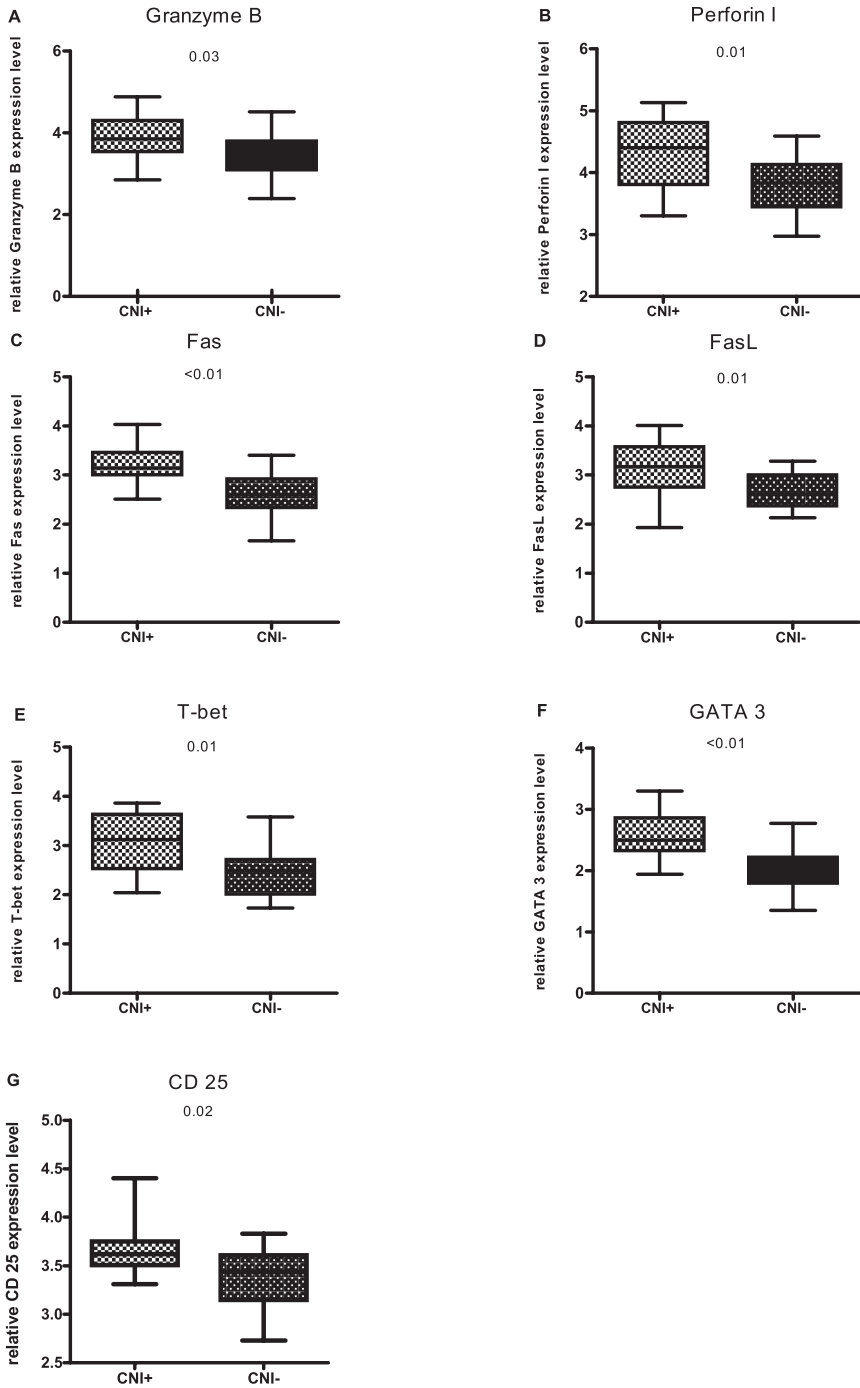
FIGURE 3. Analysis of relative mRNA expression levels before (CNI+) and 4 months after (CNI-) withdrawal. The values are log transformed. Figures A and B are represent the markers of cytotoxicity (Perforin I, Granzyme B), figures C and D the apoptotic markers (Fas and FasL), figure E and F the Th1 (T-bet) and Th2 (GATA 3) transcription factors, and figure G the IL-2 receptor CD25.

Relative mRNA expression levels before and after CNI withdrawal
Figure 3 shows significantly lower mRNA levels of granzyme B, perforin, Fas, FasL, T-bet and GATA3 in PBMC patients after CNI discontinuation. This effect was also seen for the CD25 mRNA expression levels.
No effect of CNI withdrawal was found for the mRNA expression levels of FOXP3 (p=0.10, figure 4A), IL-10, RORγt and IL-17 (data not shown).

mRNA expression levels vs donor-specific cytotoxicity
To define the relationship between donor-specific cytotoxicity and gene expression profiles, we compared the mRNA expression levels of PBMC from patients with and without a detectable donor specific CTLpf (> $10/10^6$ vs <$10/10^6$ PBMC). Before CNI discontinuation, no significant difference in mRNA expression level of granzyme B, perforin, Fas, FasL, T-bet, IL-2, GATA3, IL-10, RORγt, IL-17, FOXP3 and CD25 was found between patients with and without detectable donor-specific CTLpf. After CNI withdrawal, a significant difference between FOXP3 mRNA transcription levels in patients with (N=5) and without (N=13) donor specific CTLpf was found. This transcription factor for regulatory T-cells discriminated between detectable and non-detectable donor-specific cytolytic reactivity. The highest FOXP3 mRNA levels were measured when donor-specific cytotoxic reactivity was not measurable (Figure 4B, p<0.01).
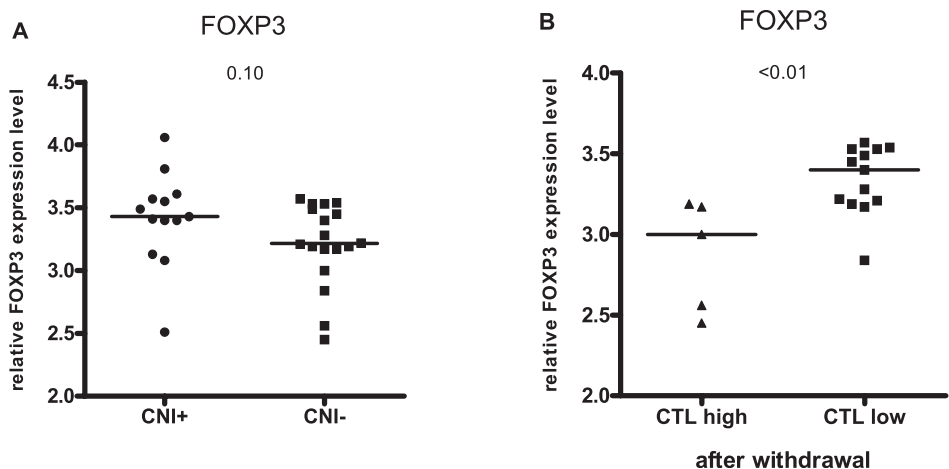
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FIGURE 4. (A) Analysis of FOXP3 mRNA expression levels before (CNI+) and 4 months after (CNI-) withdrawal . (B). Relative FOXP3 mRNA expression in patients with high donor specific CTLpf (≥$10/10^6$ PBMC) vs low donor-specific CTLpf (< $10/10^6$ PBMC) after CNI withdrawal.

Discussion

The present study shows that stable kidney transplant patients on CNI, MMF and steroids > 2 years after transplantation can be safely withdrawn from CNI. The avoidance of rejection and the achievement of clinical stability in renal function were ascertained even with further tapering of MMF of 25% at the 4th and at the 8th month. In contrast to studies showing an effect on CNI withdrawal shortly after transplantation in our patients, in whom the CNI was stopped later after transplantation, no effect of CNI withdrawal was observed on kidney function parameters, blood pressure and cholesterol [12]. These findings suggest that long-term treatment with CNI, affect the mechanisms and cells in CNI treated patients in such a way that damage occurred and as a result no beneficial effect of CNI withdrawal can be found. The mechanisms by which CNI treatment induces side effects are not completely understood but it is known that for instance the CNI CsA increases mRNA levels of a number of genes involved in cholesterol biosynthesis and hypertension [15, 16].

Apart from the parameters for kidney function, blood pressure and cholesterol, we studied the effect of CNI withdrawal on several immune biomarkers. The patients' material available allowed us to perform assays only 4 months after the CNI withdrawal and not during the period of the further MMF's tapering. CNI withdrawal significantly decreased the mRNA expression levels in unstimulated PBMC for granzyme B, perforin, Fas, FasL, T-bet, GATA3 and CD25. Furthermore, a decrease in donor-specific and not anti-third party CTLpf was measured. The latter findings in combination with the decreased granzyme/perforin levels imply a direct and specific down-regulation or deletion of anti-donor CD8+ cytotoxic T cells after withdrawal of CNI. It has been demonstrated that FoxP3+ regulatory T cells control antigen activated CD8+ effector T cells. In a mouse model, depletion of CD4+FoxP3+ regulatory T cells before viral infection significantly enhanced the magnitude of virus-specific CD8+ T cell effector function [17]. Moreover, data from human regulatory T cells show that CD4+FoxP3+ cells also modulate the number, activation and function of CD8 T cells [18]. This mechanism of action of FoxP3+ T cells could be the explanation our findings. Circulating activated donor-specific CD8+ T cells are not properly controlled by CD4+FoxP3+ T cells in CNI treated patients. CNIs interfere in the mechanisms that contribute in diminishing the anti-donor repertoire [19]. CNI block the phosphatase activity of calcineurin, which is then unable to dephosphorylate NFAT, remains in the cytoplasm and therefore prevents the transcription of IL-2 [20-23]. IL-2 is well known for its ability to promote T-cell proliferation, to inhibit apoptosis and to induce cytokines like IFN-γ. Moreover IL-2 regulates granzyme B and perforin expression [24]. The importance of IL-2 in the function of cells that control immune reactivity, the FoxP3+ regulatory T-cells, is well recognized [11, 25]. Furthermore, others and we have shown that CNI prevent the transcription of FoxP3 and a loss of the highly suppressive CD27+ regulatory T cell population has been reported [10, 26]. Here we confirm that *in vivo*, in kidney transplant patients indeed CNI interfere in

the cascades leading to donor –specific hyporesponsiveness. In the absence of CNI, cytotoxic hyporesponsiveness was associated with high FOXP3 mRNA expression levels and suggests that donor-specific reactivity is the result of impaired regulation by FOXP3+ T-cells. After withdrawal of CNI, these regulatory mechanisms emerge. Our study shows that CNI withdrawal in stable kidney transplant recipients who are at least 2 years after transplantation is safe but in contrast to withdrawal at earlier time point after transplantation, not associated with reduction in CNI related side effects. In addition, discontinuation of CNI treatment allows the development of FOXP3+ regulatory T-cells resulting in a significant decrease of anti-donor immune reactivity.

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Chapter 7

Successful tapering of immunosuppression to low dose monotherapy steroids after living-related HLA-identical renal transplantation

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Abstract

Introduction
Living-related HLA-identical renal transplant (RTx) recipients often receive standard immunosuppression, despite the absence of mismatched major HLA-antigens and the known complications of long-term use of immunosuppression. No data are available on the need for immunosuppression for these specific patients. We wondered whether their immunosuppressive load could be radically reduced.

Method
Between November 1982 and November 2005, 83 living-related HLA-identical RTx were performed in our center. Their unadjusted graft survival was 74% at 10 years. In 29 patients (median time after transplantation 5.6 (range 1.0-21.4) years) with stable uncompromised renal function, we tapered their immunosuppression from triple or dual therapy to prednisolone 5 mg/day. Follow up on prednisolone monotherapy was at least 24 months.

Results
In 27 of 29 patients reduction of immunosuppression to prednisolone monotherapy was uneventful. One patient, using dual therapy, developed JC-virus nephropathy resulting in graft loss. One refused further discontinuation of his medication. Four (15%) of the 27 patients on monotherapy developed biopsy proven recurrence of their original disease. Only one of them showed a transient decline in renal function. One additional patient developed minor proteinuria and a rise in serum creatinine level, due to chronic urinary tract infections. The remaining 23/27 (85%) patients had an uneventful follow up during 24 months prednisolone monotherapy.

Conclusion
We conclude that HLA-identical living-related RTx recipients who are at least one year after transplantation might be treated with low dose steroid monotherapy. Close surveillance of patients for recurrence of their original disease is recommended to allow for potential early therapeutic intervention.

Introduction

After Human Leucocyte Antigen (HLA)-identical living-related (LR) renal transplantation (RTx) there is less acute rejection and better graft survival compared with non-identical renal transplant recipients. These superior results are due to the fact that HLA-identical LR transplants are less immunogenic than non-identical renal transplants, because in HLA-identical LR RTx all major (class I and II) HLA molecules are identical and only mismatches in minor histocompatibility antigens (mHAgs) or non-HLA antigens may exist. In animal models, the importance of mHAgs has been shown after cardiac transplantation and allogeneic bone marrow transplantation(1-5). In humans, mismatches in mHAgs have been shown to induce graft versus host disease (GVHD) after HLA-identical bone marrow transplantation, but minor HLA mismatches had no influence on 5-year graft outcome after RTx (6, 7).
Nevertheless, recipients of HLA-identical LR donor kidney generally receive the same immunosuppressive regime as HLA-mismatched renal transplant recipients. Therefore they remain at risk for cardiovascular disease, metabolic complications, infections and malignancies, all known side effects of immunosuppressive medication and familiar risk factors for poor patient and graft survival after RTx(8).
Insufficient data are available about immunosuppression after HLA-identical LR RTx (9-13). Even less data are available of the possibility to reduce or discontinue their immunosuppressive medication(14). Considering this, we designed a study to reduce the immunosuppressive load dramatically in this specific patient group.

Materials and methods

Patients
Between November 1982 and November 2005, 83 LR HLA-identical RTx were performed in our center. Molecular HLA typing was performed on DNA obtained from blood by polymerase chain reaction/sequence-specific oligonucleotide using a reverse dot-blot method. (20) Al study patients who were transplanted before this technique was available were retyped with this PCR technique to be sure they were really HLA-identical with their donor. A transplant was classed as HLA-identical if donor and recipient were reported to have identical HLA A, B, Cw, DR and DQ antigens. All patients had negative cross matches with their donor prior to transplantation. Of these 83 patients, 43 (52%) were male and 40 (48%) were female. Their median age was 50 yrs (range 21-78 yrs)(Table 1). At time of observation (n=83), the median time after transplantation was 7.0 years (range 0.8-23.8 yrs). Their unadjusted graft survival was 74% at 10 years, compared to 61% after LR HLA-mismatched renal transplantation (Figure 1).

	Total HLA- identical RTx	Not enrolled	Study Group
Number of patients	83	54	29
Male: female	43:40	28:26	15:14
Age **(median in yrs, range)**	50 (21-78)	50 (26-78)	51 (21-66)
Male recipient, female donor **Female recipient, male donor**	28/43 (65%) 17/40 (43%)	18/28 (64%) 11/26 (42%)	10/15 (67%) 6/14 (43%)
Time after RTx **(median in yrs, range)**	7.0 (0.8-23.8)	8.8 (0.8-23.8)	5.6 (1.0-21.4)
Original disease with potential **to recur**	42 (51%)	26 (49%)	16 (55%)

Table 1: baseline characteristics of the living-related HLA-identical renal transplant recipients transplanted between November 1982 and November 2005.
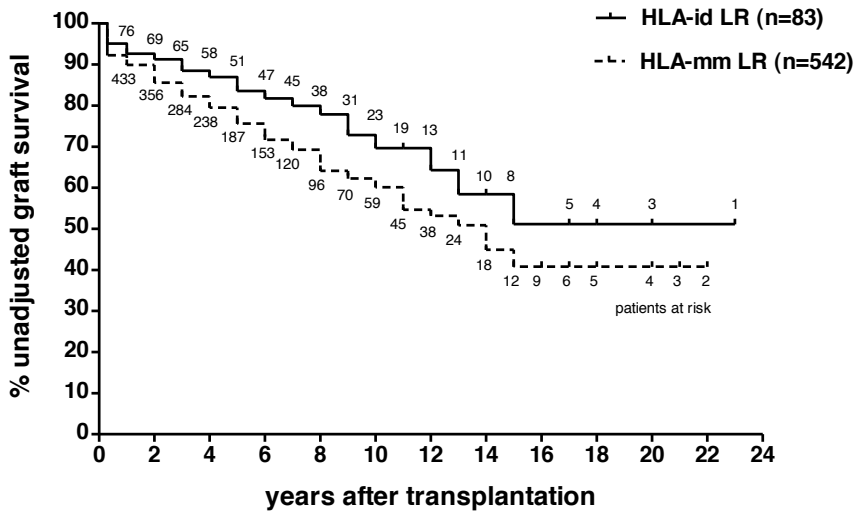
Figure 1: Unadjusted graft survival after living-related HLA-identical (solid line) and HLA-mismatched (dotted line) renal transplantation

Out of 83 HLA-identical LR RTx recipients, 54 patients could not be enrolled in our study (demographics see Table 1). Seven (13%) patients died before with a function-ing kidney (median time after RTx was 9.6 yrs, range 0.9-15.6 yrs), due to cardio-vascular disease (n=4), infection (n=1), malignancy (n=1) and suicide (n=1).
In 6 (11%) patients graft loss was observed, due to recurrence of the original disease (n=1), chronic rejection (n=2), tubular interstitial nephritis due to medication (n=1), surgical complication (n=1) and infection (n=1).

Two (4%) patients were less then 1 year after transplantation, 3 (6%) patients received a kidney from their identical twin sister, 1 (2%) patient already used monotherapy, in 1 (2%) patient was transplanted for the 4th time, 11 (20%) patients were excluded for logistic reasons and 7 (13%) refused to participate in the present study. Seventeen (31%) patients could not be included because of proteinuria. Of these 17 patients, 6 patients had a biopsy proved recurrence of their original disease, 5 patients had a chronic allograft nephropathy (CAN), in 1 patient the proteinuria disappeared after nephrectomy of his native kidneys and in 5 patients the cause of their proteinuria remained unknown.

The ethical review committee of our center approved the protocol that was conducted according to local requirements. After informed consent, 29 LR HLA-identical renal transplant recipients who were more then 1 year after RTx, with stable renal function, without proteinuria (<0.2 g/l) and on triple or dual immunosuppression were enrolled in our immunosuppression reduction study. Their demographics are shown in table 1. The median time after transplantation was 5.6 years. Only 5 patients were more than 10 years after transplantation.

Depending on the medication patients used at time of inclusion, we started tapering their calcineurin inhibitor (CNI), followed by mycophenolate mofetil (MMF) or azathioprine (AZA) and prednisolone dose with 2 months regular intervals to prednisolone monotherapy of 5 mg/day. Serum creatinine levels and proteinuria were monitored. A renal biopsy was taken if patients developed a clinically relevant rise of serum creatinine or proteinuria (defined as >0.5 g/l). Blood was obtained for monotoring T-cell reactivity (results described by Gerrits et al, submitted to Transplantation)

Statistical methods
Data for this study were obtained by patient chart analysis. Survival curves were made using the Kaplan-Meier method and the log-rank test was used to compare the survival rates. Continuous variables are reported as means ± SD and tested by paired Student's t-test. Data that did not follow a normal distribution are presented as medians and tested by Wilcoxon signed rank test. Qualitative variables are reported as percentages and were tested by the Pearson's chi-squared test.

The SSPS statistical package version 12.0.1 was used. P-values <0.05 were considered significant

Results
Twenty-nine HLA-identical LR RTx recipients were included in our immunosuppression reduction study. In 27 patients this was their first, for 1 patient it was his second and for 1 it was her third RTx. Their median PRA was 2% (range 0-98%) before transplantation.

The majority of the patients used dual immunosuppressive therapy at inclusion. Nineteen (66%) used AZA combined with prednisolone, 4 (14%) patients used tacrolimus

(Tacro) combined with mycophenolate mofetil (MMF), 3 (10%) patients used MMF combined with prednisolone and 1 (3%) patient used cyclosporine (CyA) combined with prednisolone. Two patients (7%) used triple immunosuppression consisting of Tacro, MMF and prednisolone. Figure 2 shows the results of serum creatinine levels and proteinuria at time of inclusion (using different combinations of immunosuppressive medication), at the moment patients started with prednisolone monotherapy 5 mg/day and after they had been on prednisolone monotherapy for 12 and 24 months.

■ Patient 7: recurrence Membranous GN, 4.6 yrs after RTx
▲ Patient 11: Chronic Pyelonephritis, 3.3 yrs after RTx
∗ Patient 13: JC-virus infection, 1.1 yrs after RTx
O Patient 21: recurrence DM, 10.7 yrs after RTx
● Patient 24: recurrence Membranous GN, 7 yrs after RTx
♦ Patient 29: recurrence IgA nephropathy, 7.8 yrs after RTx

Figure 2: Serum creatinine levels and proteinuria at time of inclusion (using different combinations of immunosuppressive medication), at the moment patients started with prednisolone monotherapy 5 mg/day and after they had been on prednisolone monotherapy for 12 and 24 months.
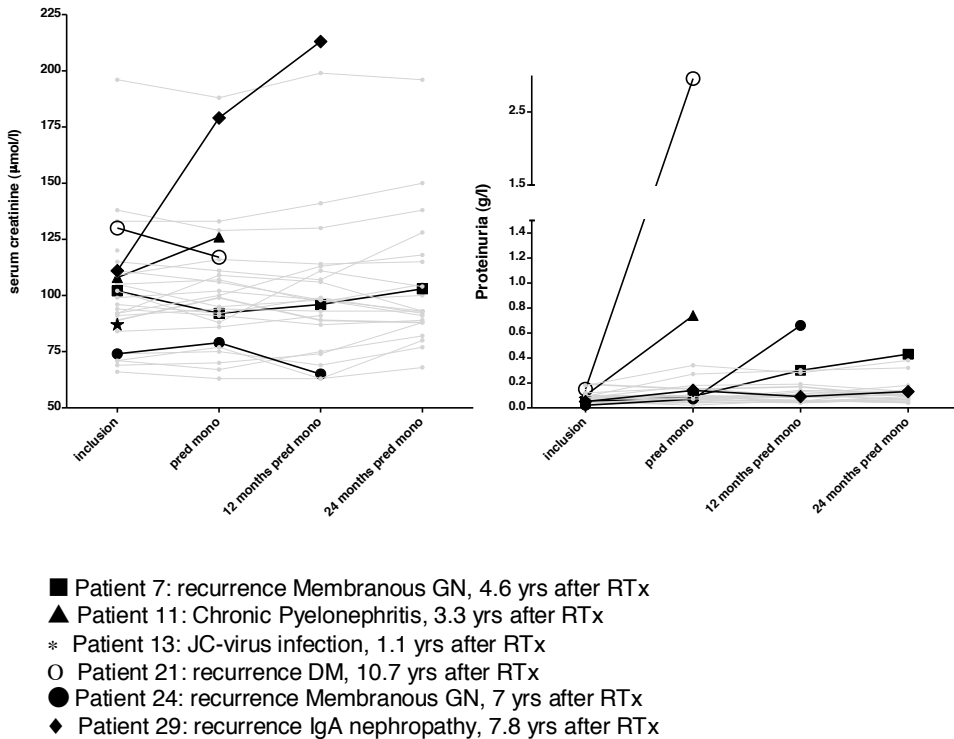
One patient refused further discontinuation of his immunosuppressive medication after inclusion in the study. Another patient, still on dual therapy, developed a biopsy proved JC-virus nephropathy 13.3 months after transplantation. Despite reducing her immunosuppressive medication to prednisolone 10 mg/day, combined with leflunomide 30 mg/day, she had a progressive decline of her creatinine clearance resulting in graft loss 25.3 months after RTx.

Four (15%) of the 27 patients showed a recurrence of their original disease in their renal biopsy, after being on monotherapy prednisolone 5 mg/day for 2, 13, 17 and 22 months and 11, 8, 5 and 7 years after RTx respectively. Despite proteinuria, serum creatinine levels remained stable in 3 of them. The other showed a transient rise in serum creatinine level, due to IgA nephropathy, which stabilized after reintroduction of MMF 2 gram a day and raising the prednisolone dose to 10 mg/day. Another patient showed a rise in serum creatinine level due to a chronic urinary tract infection, after several urological procedures (Table 2).

Patient	Serum Creatinine (μmol/l)	Protein-uria (g/l)	Medication	Years after RTx	Diagnose
7	Stable (102→103)	+ (1.5)	Pred mono for 16.8 months	4.6	Recurrence membranous nephropathy
11	↑ (108→126)	+ (0.74)	Pred mono for 2 months	3.3	Chronic urinary tract infection
13	↑ (87→213)	- (0.02)	MMF 500 mg + Pred 5 mg	1.1	JC-virus infection
24	Stable (58→64)	+ (1.73)	Pred mono for 21.7 months	7.0	Recurrence membranous nephropathy
21	Stable (133→107)	+ (2.96)	Pred mono for 2.1 months	10.7	Recurrence diabetes nephropathy
29	↑ (111→203)	- (0.09)	Pred mono for 13.3 months	7.8	Recurrence IgA nephropathy

Table 2: Characteristics of patients who developed proteinuria or a rise in their serum creatinine during tapering of their immunosuppressive medication. Clinical relevant proteinuria is defined as > 0.5g/l. "Medication" is defined as the medication patients used at the moment their serum creatinine rise and/or they developed proteinuria. Diagnose is made on clinical grounds and confirmed by renal biopsy. "Pred"=prednisolone, "MMF"=mycophenolate mofetil, "RTx"=renal transplantation

In 23 (85%) of the 27 patients the immunosuppression could be successfully reduced to prednisolone monotherapy 5 mg/day. No significant changes in serum creatinine levels, 99 μmol/l (range 66-196) vs. 93 μmol/l (range 68-196), or protein excretion, 0.08 (range 0.02-0.19) vs. 0.10 (range 0.04-0.38) g/l, were observed between time of inclusion and after 24 months of prednisolone monotherapy 5 mg/day. There were no significant changes in systolic-, diastolic blood pressure, the number of antihypertensive drugs taken, serum total-, LDL- or HDL cholesterol levels, haemoglobulin, thrombocytes or leucocytes between time of inclusion and after 24 months of prednisolone monotherapy.

Discussion

With the current results of patient and graft survival after RTx we are confronted with the inherited complications of long-term use of immunosuppressive medication. Therefore, we have the obligation to investigate the possibilities of tapering this medication without reducing the short and long-term graft and patient survival. A lot of our study patients were treated with azathioprine and prednisolone, by many classified as "light immunosuppression". These patients were probably at low risk for rejection after tapering immunosuppression and this is exactly what we showed. More over we should keep in mind that even maintenance therapy with so-called "light immunosuppression" is accompanied with serious side effects.

 In our opinion, in living-related HLA-identical RTx recipients monotherapy with low dose steroids, although not devoid from all side effects, is preferred above monotherapy with low dose AZA, MMF or CNI, with regard to infections and malignancies. This also holds true for recently described protocols with antithymocyte globulin, total lymphoid irradiation and hematopoietic-cell transplantation (15). In our LR HLA-identical RTx study group, dramatically tapering of their immunosuppressive medication to low dose prednisolone monotherapy is well tolerated, without the occurrence of acute rejections during a follow up of 2 years. Acute rejection episodes after identical sibling RTx have been reported. However, the majority of them were described in the AZA era in a time class II match was not perfect and BK-virus nephropathy was an unrecognized entity. Nevertheless, mismatches in minor HLA-antigens has been found relevant in the context of bone marrow transplantation and might theoretical induce immunological reactivity against minor mismatched solid organs. Recently, Gerrits et al described in vitro reactivity against donor cells after HLA-identical living-related RTx, but could not prove that this was the result of mismatches in minor HLA-antigens(16). Thus, acute rejection after tapering immunosuppression could be immunologically explained. However, it did not occur in our study, which is in line with the observation of Heinold et al, who did not find a clinical relevant role of minor HLA mismatches after solid organ transplantation (7). Differences in non-HLA antigens could be an alternative explanation for donor reactivity after HLA-identical RTx (18,19)

After tapering their immunosuppressive medication, recurrence of original disease occurred in 15% (4 out of 27) of the patients of the total study group, or otherwise specified, in 25% (4 out of 16) of the group of patients who had an original disease with potential to recur. After 2 years follow up, none of their renal grafts had failed. It should be mentioned that 1 of these 4 patients had a diabetes nephropathy. We wondered whether the recurrence of primary glomerulonefritis in the other 3 patients could be related to the tapering of their immunosuppressive load or that this just reflects the natural course of recurrence of a primary glomerulonephritis after RTx. In 1999, Andresdottir et al described a biopsy proven prevalence of recurrence of original disease after LR HLA-identical RTx of at least 27%, with a graft failure due to

recurrence of 15%, with a mean time after transplantation of 7.7 ±6.1 years (17). Before we embarked on the present study, we screened our LR HLA-identical population under full dose immunosuppression. There was a prevalence of 17% (7 out of 42) of biopsy proven recurrence of original disease, in the group of patients who had that potential. In 5 patients with proteinuria, no histology was available, so the true incidence of recurrence could have been as high as 35%. This suggest that the prevalence of recurrence after tapering immunosuppressive medication in our study group was comparable to that described before in LR HLA-identical RTx recipients who used full dose immunosuppression and is in line with earlier observation.

In conclusion, the immunosuppressive medication can be safely reduced to low dose steroid monotherapy of 5 mg/day in HLA-identical living-related renal transplant recipients provided that they have stable renal function, without proteinuria and they are at least one year after transplantation. Close surveillance of patients for recurrence of their original disease is recommended to allow for potential early therapeutic intervention.

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Chapter 8

T-cell reactivity during tapering of immuno-suppression to low dose monotherapy prednisolone in HLA-identical living-related renal transplant recipients

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Abstract

Background

In many transplant centers, HLA-identical living-related (LR) renal transplant recipients receive standard maintenance immunosuppression from one year after transplantation. We questioned whether discontinuation of AZA or MMF influenced T-cell reactivity, circulating DC subsets numbers and their maturation status.

Methods

Twenty-nine HLA-identical LR renal transplant recipients were withdrawn from AZA or MMF. Thereafter, the patients received only prednisolone. T-cell reactivity was determined by IFN-γ (n=23), IL-10 (n=16) and granzyme B (GrB; n=10) Elispot assays. Circulating DC subset numbers and their maturation status determined by CCR2, CCR5, CCR7 and CD83 expression were measured by flow cytometry (n=12).

Results

The number of donor, 3rd-party, and TET-reactive IFN-γ and GrB producing cells was not affected after withdrawal of immunosuppression. Discontinuation of AZA or MMF resulted in significant increased numbers of 3rd-party (p=0.003) and TET-reactive (p=0.008) IL-10 producing cells, and a trend in higher numbers of donor-reactive IL-10 producing cells (p=0.06). No effect was found on the number of circulating DC subsets, but DC were shifted towards a more mature phenotype.

Conclusions

In HLA-identical LR renal transplant recipients, therapy with AZA and MMF suppress the IL-10 production and the maturation of DC. This suggests that these immunosuppressants may hinder downregulation of immune responses in general, including allogeneic responses.

Introduction

After HLA-identical LR renal transplantation, mismatches only in minor histocompatibility antigens (mHAgs) and other non-HLA antigens may exist between donor and recipient (1, 2). mHAgs are genetic inherited peptides derived from polymorphic intracellular proteins presented in the context of HLA class I and II molecules and can be recognized by T cells. In humans, the clinical relevance of mHAgs has been reported after bone marrow transplantation (2), and even after solid organ transplantation a role of mHAgs and non-HLA antigens has been suggested (3-5). Theoretically, both mismatches in mHAgs and other non-HLA antigens between donor and recipient might induce graft rejection. Consequently, HLA-identical LR renal transplant recipients still receive immunosuppression. However, the necessity for long-term use of immunosuppression in this patient group is yet unknown. We wondered whether HLA-identical LR renal transplant recipients should be exposed to the severe side-effects of immunosuppression such as nephrotoxicity, malignancies, cardiovascular disease and diabetes mellitus (6, 7).

Previously, we demonstrated that donor-reactive cytokine responses can be found after HLA-identical LR renal transplantation (8). Additionally, we showed that HLA-identical LR renal transplant recipients, who received azathioprine (AZA) in combination with prednisone, could be tapered to 50% of their original AZA dose without the occurrence of acute rejection. Furthermore, renal function and donor-reactive responses remained unaffected after tapering the AZA dose (9, 10). Therefore, we speculate that those patients are still over-immunosuppressed and that the immunosuppressive dose could be reduced further.

Increased donor-reactivity could occur after discontinuation of immunosuppression in transplant recipients after HLA-mismatched renal transplantation (11-14). Complete discontinuation of immunosuppression has been reported in a minority of renal transplant recipients long after transplantation with stable graft function and without clinical signs of rejection (15-20). In general, a decreased T-cell response was reported in those studies compared to patients with chronic allograft nephropathy (15, 16, 19, 20).

In addition to immunological monitoring of donor-reactive T-cell responses in transplant recipients, it has been suggested that monitoring of circulating myeloid dendritic cells (CD11c^{+}CD123^{low}BDCA-1^{+} mDC) and plasmacytoid DC (CD11c^{-}CD123^{high}BDCA-2^{+} pDC) (21) numbers in peripheral blood might be an useful tool for identifying transplant recipients in whom the immunosuppressive load can be safely tapered (22, 23). mDC produce high levels of IL-12 and induce T-helper 1 (Th1) and cytotoxic T-cell (CTL) responses, while pDC produce IFN-α in response to viruses and induce T-helper 2 (Th2) responses (24). Furthermore, it has been suggested that pDC are involved in the induction of peripheral T-cell tolerance after organ transplantation (23,

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25). According to their surface immunophenotype, DC subsets can be identified as immature DC and mature DC (24, 26). In peripheral blood and tissues, DC reside as immature DC where they may internalize antigens. Upon antigen capture, immature DC differentiates into mature DC that are highly specialised to stimulate T cells efficiently (27). Several studies reported the influence of immunosuppressive drugs on DC subset numbers, differentiation and their maturation status (23, 28-33). Furthermore, Mazariegos et al. reported that the proportion of pDC in peripheral blood mononuclear cells (PBMC) was higher in stable liver transplant recipients who could be successfully weaned from their immunosuppressive load (23).

In the present study, we discontinued the AZA and mycophenolate mofetil (MMF) dose. Thereafter, all patients received at least one-year steroid monotherapy. We questioned whether discontinuation of AZA or MMF influenced T-cell reactivity determined by Elispot assays. This assay was used to determine the frequency of pro-inflammatory cytokine IFN-γ and anti-inflammatory cytokine IL-10 that have been associated with allograft rejection or downregulation of the immune response, respectively (34, 35). Granzyme B (GrB) was used as a marker of activity of cytotoxic T-lymphocytes (CTL) (10, 36). CTL plays a crucial role in allograft rejection (37). Additionally, we wondered whether discontinuation of immunosuppression affected the circulating DC subsets numbers and their maturation status determined by flow cytometry.

Materials and Methods

HLA-identical LR renal transplant recipients

The ethical review committee of our center approved the protocol that was conducted according to local requirements. Between November 1982 and November 2005, 83 living-related HLA-identical renal transplants were performed in our center. Out of those 83 patients, 54 patients could not be enrolled in our study: 7 patients died with a functioning kidney (n=4: cardio-vascular disease; n=1: infection; n=1: malignancy; n=1: suicide), in 6 patients graft loss was observed (n=1: recurrence of original disease; n=2: chronic rejection; n=1: tubular intestinal nephritis due to medication; n=1: surgical complication; n=1: infection), 2 patients were less than 1 year after transplantation, 3 patients received a kidney from their HLA-identical twin sister, 1 patient already used monotherapy, 1 patient was transplanted for the 4th time, 11 patients were excluded for logistic reasons, 7 patients refused to participate in the present study, and 17 patients could not be included because of proteinuria (n=6: recurrence of their original disease; n=5: chronic allograft nephropathy; n=1 proteinuria disappeared after nephrectomy of his native kidneys, n=5: cause of proteinuria is unknown)(57). After informed consent, 29 HLA-identical LR renal transplant recipients agreed to participate in this study. Characteristics of the patients are described in Table 1.

Table 1: Characteristics of the HLA-identical living-related renal transplant recipients

Patient	Gender [1]P	Gender [2]D	Primary disease	Age at RTx (years)	T_{DT}[3] (years)	# RTx[4]	IS[5] (+prednisolone 5 mg/day)	Reached mono-therapy?	Tested in Elispot assays?	Analysed by flow cytometry?
1	M	M	Focal segmental glomerulosclerosis	41.5	1.6	1	MMF[8]	no	-[10]	-
2	F[6]	M[7]	AL-amyloïdose	46.9	1.7	1	AZA[9]	yes	+[11]	+
3	F	F	Rapidly progressive glomerulonephritis	62.0	4.4	1	AZA	yes	+	-
4	F	F	Focal segmental glomerulosclerosis	11.8	12.3	1	AZA	yes	+	-
5	M	M	Focal segmental glomerulosclerosis	34.7	4.9	1	MMF	yes	+	+
6	M	M	Membranous glomerulonephritis	48.9	7.1	1	AZA	yes	+	+
7	M	F	Membranous glomerulonephritis	52.2	3.0	1	MMF	yes	+	+
8	M	M	Hypertension	31.1	13.6	1	AZA	yes	+	+
9	F	M	Chronic pyelonephritis	37.6	22.0	1	MMF	yes	+	+
10	F	F	Medullary cystic disease	54.8	2.9	1	AZA	yes	+	+
11	F	F	Congenital obstructive nephropathy	25.2	3.9	1	MMF	yes	-	-
12	M	F	Acute tubular necrosis	21.3	6.8	1	AZA	yes	+	+
13	F	M	Polycystic kidney disease	51.4	1.0	1	MMF	no	-	-
14	M	F	Membranous glomerulonephritis	33.6	11.2	1	AZA	yes	+	+
15	F	F	Unknown	46.2	17.3	1	AZA	yes	+	-
16	M	M	IgA nephropathy	48.6	6.7	1	AZA	yes	+	+
17	M	F	Hypertension	38.5	2.5	1	MMF	yes	+	-
18	F	M	SLE nephropathy	41.2	9.0	1	AZA	yes	+	+
19	M	F	Von Hippel Lindau	35.5	6.3	1	AZA	yes	+	+
20	F	F	IgA nephropathy	46.0	5.4	3	MMF	yes	+	-
21	M	F	Diabetes nephropathy	41.5	10.5	1	MMF	yes	-	-
22	M	F	Necrotic glomerulonephritis eci	19.2	2.3	1	MMF	yes	+	+
23	F	F	Meningococcal sepsis	51.1	4.4	1	AZA	yes	+	-
24	F	M	Extracapillary glomerulonephritis	50.9	5.1	1	AZA	yes	+	-
25	F	F	Chronic pyelonephritis	43.7	12.5	1	AZA	yes	-	-
26	M	M	Adult polycystic kidney disease	39.1	7.2	1	AZA	yes	+	-
27	M	F	Reflux nephropathy	47.8	5.4	2	AZA	yes	+	-
28	F	M	Diabetes Mellitus II and hypertension	56.7	5.6	1	AZA	yes	+	+
29	M	F	IgA nephropathy	52.3	6.6	1	AZA	yes	-	-
median				43.7	6.3	1				

[1]P, patient; [2]D, donor; [3]T_{DT}, time from transplantation to inclusion of study (dual therapy: DT); [4]RTx, first, second or third renal transplantation; [5]IS, immunosuppressive medication at dual therapy; [6]F, female; [7]M, male; [8]MMF, mycophenolate mofetil; [9]AZA, azathioprine; [10]-, not determined; [11]+, determined.

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The patients were more than one year after transplantation with stable serum creatinine levels and no proteinuria (<0.5 g/L), and on triple or dual immunosuppressive therapy. From the 29 patients, 19 patients used AZA in combination with prednisolone and 3 patients used MMF in combination with prednisolone. The other 7 patients (n=4, Tacro+MMF; n=1, CsA+prednisolone; n=2, Tacro+MMF+prednisolone) were converted to 500 mg/day MMF in combination with 5 mg/day prednisolone. Then, the AZA or MMF dose was gradually discontinued over a period of 4 months and patients were kept on 5 mg/day prednisolone monotherapy. The follow-up of the patients on prednisolone monotherapy was one year (Figure 1). Our laboratory analysis on T-cell reactivity started at dual therapy and 1-year monotherapy (Figure 1).
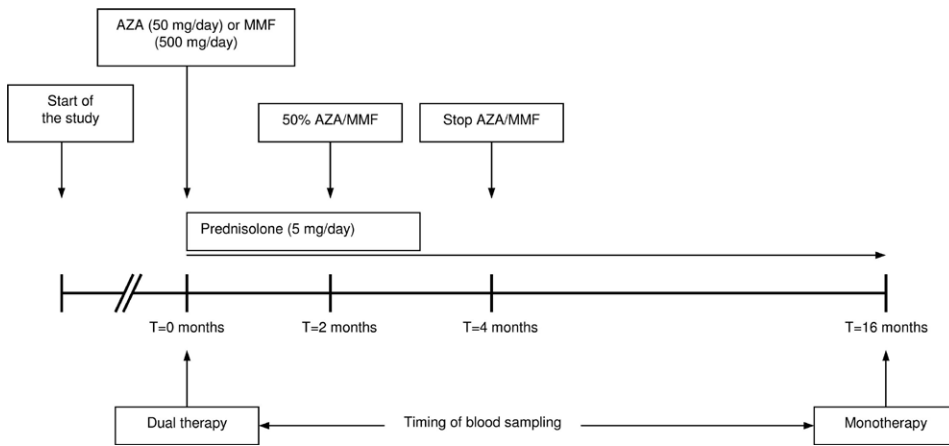

Figure 1:
Schematic overview of discontinuation of immunosuppressive medication in HLA-identical living-related renal transplant recipients and time of blood sampling (dual therapy and 1-year steroid monotherapy).

Blood sampling
We received 35 ml heparinized peripheral blood at dual therapy and monotherapy to perform Elispot assays (33 ml peripheral blood), and to measure DC subset numbers and their maturation status (2 ml peripheral blood). PBMC from recipient and donor were isolated from heparinized blood by density gradient centrifugation using Ficoll-Paque (Amersham Biosciences, Uppsala, Sweden) and stored at –140°C as described before (9).

IFN-γ, IL-10 and GrB Elispot assays
The phytohemagglutinin (PHA; Murex Biotech, Kent, UK) proliferation assay was performed to control the viability of the PBMC as described before (9). The mean counts per minute (cpm) were determined, and the stimulation index (SI) was calculated by the ratio of the cpm obtained in the presence of PHA to the cpm in the absence of PHA. Only results of viable cells (SI $\geq$ 50) were analyzed in the described results.

The IFN-γ, IL-10 and GrB Elispot assays (U-CyTech Biosciences, Utrecht, The Netherlands) were used to determine the number of cytokine producing cells reactive to donor, 3rd-party and tetanus toxoid (TET) antigens (38). Briefly, in a 96-wells round bottom plate (Nunc, Roskilde, Denmark), patients' PBMC were stimulated with donor PBMC, 3rd-party PBMC and TET. The 3rd-party PBMC did not share HLA antigens with donor and patient, and the same 3rd-party PBMC was used at dual therapy and steroid monotherapy. TET stimulation (RIVM, Bilthoven, The Netherlands) was used to determine the memory immune response to nominal antigens. As negative controls, we used patients' PBMC stimulated with irradiated patients' PBMC (autologous response; to control the influence of irradiation), and patients' PBMC in culture media alone (unstimulated patients' PBMC). The autologous response was subtracted from the donor and 3rd-party reactive response. The response of unstimulated patients' PBMC was subtracted from the TET response. After 40 hours of incubation, non-adherent cells were harvested and transferred in triplicate to a flat-bottom plate (Nunc, Roskilde, Denmark) precoated with IFN-γ, IL-10 or GrB monoclonal antibodies (U-CyTech Biosciences) and post-coated with phosphate-buffered saline (PBS) supplemented with 1x Blocking stock solution B (U-CyTech Biosciences). Cells were incubated for 5 hours at 37°C for IFN-γ and GrB Elispot assays, and overnight for IL-10 Elispot assay. Detection of spots was performed as described before (38). The spots were counted automatically by using a Bioreader 3000 Elispot reader (BioSys, GmbH, Karben, Germany).

Antibodies for mDC and pDC staining
Fluorescence-activated cell sorter (FACS) analysis was performed using the following mouse anti-human monoclonal antibodies: allophycocyanin (APC)-conjugated BDCA-1 (clone: AD5-8E7) and APC-conjugated BDCA-2 (clone: AC144) (Miltenyi Biotec, GmbH, Germany), peridinin chlorophyll protein (PerCP)-conjugated CD14 (clone: mφP9) and PerCP-conjugated CD19 (clone: 4G7; Becton Dickinson Biosciences, San Jose, CA, USA), fluorescein isothiocyanate (FITC)-conjugated CD83 (clone: HB15A17.11; DPC, Serotec, Oxford, UK), phycoerythrin (PE)-conjugated CCR2 (clone: 48607.211) and PE-conjugated CCR7 (clone: 150503; R&D Systems Europe, Abingdon, UK), FITC-conjugated CCR5 (clone: 2d7; Becton Dickinson), and FITC-conjugated IgG2a (clone: X39) and PE-conjugated IgG2b (clone: X39) isotype control monoclonal antibodies (Becton Dickinson).

Immunofluorescence staining and flow cytometric analysis of dendritic cell subsets and their maturation status
Analysis of DC numbers and maturation status was performed as described before (39). Briefly, 2 ml fresh heparinized blood was obtained from 12 HLA-identical LR renal transplant recipients (patient: 2, 5, 6, 7, 8, 9, 10, 14, 17, 19, 22, 28; Table 1) and processed within 4 hours. Whole blood samples were incubated with the above-

mentioned monoclonal antibodies for 30 minutes in the dark at room temperature. Cells that stained negative for CD14 and CD19 were gated and analysed for BDCA-1 and BDCA-2 expression. mDC and pDC were identified as CD14$^-$CD19$^-$BDCA$^-$1$^+$ cells and CD14$^-$CD19$^-$BDCA$^-$2$^+$ cells, respectively. Immature mDC were defined as CD83$^-$CCR7$^-$CCR5$^+$CCR2$^+$, and mature mDC as CD83$^+$CCR7$^+$CCR5$^-$CCR2$^-$. Immature pDC were defined as CD83$^-$CCR7$^+$CCR5$^+$CCR2$^+$, and mature pDC as CD83$^+$CCR7^{++}CCR5$^-$CCR2$^-$. The proportion of mDC and pDC positive for CD83, CCR7, CCR5 and CCR2 was determined by comparison to their respective isotype control antibodies. From each tube, 500,000 events were acquired on a FACScalibur flow cytometer using CELLQUEST PRO software (Becton Dickinson).

The absolute counts for each DC subset was calculated by multiplying the proportion of mDC and pDC within the total leucocyte population by the absolute number of white blood cells determined on an automatically cell counter (Casey®, Schärfe System, GmbH, Rentlingen, Germany). The absolute counts for total DC were calculated by the sum of the absolute counts of mDC and pDC. The pDC/mDC ratio was determined by dividing the absolute number of pDC with the absolute number of mDC.

Statistical analysis

The Wilcoxon signed rank test was used to compare the frequency of cytokine producing cells, the absolute number of total DC (mDC+pDC), mDC and pDC numbers, and the pDC/mDC ratio at dual therapy and monotherapy. The same test was used to compare the percentage positive of CD83, CCR7, CCR2 and CCR5 on DC subsets before and after discontinuation of AZA or MMF. The Fischer's Exact test was used to compare the number of patients that responded to TET antigens before and after withdrawal of AZA or MMF. Two sided P-values ≤0.05 were considered significant. For statistical analysis, SPSS 11.5 for Windows was used (SPSS, Inc., Chicago, IL, USA).

Results

Clinical results

After inclusion (dual therapy), 2 out of 29 (7%) HLA-identical LR renal transplant recipients did not reach steroid monotherapy due to JC-virus infection (patient 13) and one patient (patient 1) refused to discontinue his immunosuppressive medication after inclusion in the study (Table 1). None of the patients had an acute rejection episode. A detailed description of the clinical results of this study is described by Van de Wetering *et al.* (57).

Patient 11, 21, 26 and 29 (n=4) were not tested in the cellular assays, because we were unable to receive patient and donor PBMC, respectively (Table 1). We received 35 ml heparinized blood during dual therapy and during monotherapy. The PBMC yield is variable after transplantation. From all patients described below,

PBMC samples were tested during dual and after one year on monotherapy. We could perform an IFN-γ Elispot assay in 23 patients, from 16 patients enough cells were available to perform the IL-10 Elispot, and even from 10 patients we also could perform a GrB Elispot.

In 2003 it was reported that monitoring of circulating DC would be a good tool to identify transplant recipients in whom the immunosuppressive medication can be safely discontinued (23). Then, we observed that cryopreservation of PBMC significantly effects chemokine receptor markers on DC (39). In other words, we could only determine the DC subsets and their chemokine receptors in fresh whole blood. Our inclusion of patients was from the beginning of 2003. Therefore, we have monitored the DC in 12 patients before and after withdrawal of AZA or MMF.

The frequency of IFN-γ, IL-10 and GrB producing cells

The frequency of donor-reactive IFN-γ producing cells did not increase after discontinuation of AZA or MMF [dual therapy: median, 2 IFN-γ producing cells/$2x10^5$ PBMC (range, 0-13); monotherapy: median, 0 IFN-γ producing cells/$2x10^5$ PBMC (0-16); p=0.21; Figure 2A]. The donor response was significantly lower than the 3rd-party response (dual therapy, p<0.001; monotherapy, p<0.001). The 3rd-party reactivity was comparable between dual therapy [median, 33 IFN-γ producing cells/$2x10^5$ PBMC (5-333)] and monotherapy [median, 31 IFN-γ producing cells/$2x10^5$ (4-322); p=0.59; Figure 2B].

From 16 patients we were able to perform IL-10 Elispot assays. The number of donor-reactive IL-10 producing cells was low on dual therapy [median, 1 IL-10 producing cells/$2x10^5$ PBMC (range, 0-4)] and tended to be higher after discontinuation of AZA or MMF [median, 1 IL-10 producing cells/$2x10^5$ (0-80); p=0.06; Figure 2C]. No significant difference was found in donor and 3rd-party reactive IL-10 producing cells (dual therapy, p=0.18; monotherapy, p=0.06). The frequency of 3rd-party reactive IL-10 producing cells was significantly higher during monotherapy [median, 13 IL-10 producing cells/$2x10^5$ PBMC (0-208); p=0.003; Figure 2D] than during dual therapy [median, 2 IL-10 producing cells/$2x10^5$ PBMC (0-8)].

We were able to perform GrB Elispot assays from 10 patients. No difference was observed between the number of donor-reactive GrB producing cells at dual therapy [median, 2 GrB producing cells/$2x10^5$ PBMC (range, 0-40)] and at monotherapy [median, 0 GrB producing cells/$2x10^5$ (0-6); p=0.18; Figure 2E]. The donor-reactive GrB producing cells was significantly lower than the 3rd-party reactive GrB producing cells (dual therapy, p=0.008; monotherapy, p=0.007). At dual therapy, the frequency of 3rd-party reactive GrB producing cells [median, 17 GrB producing cells/$2x10^5$ PBMC (0-49)] was comparable with monotherapy [median, 16 GrB producing cells/$2x10^5$ PBMC [(0-47); p=0.84; Figure 2F].
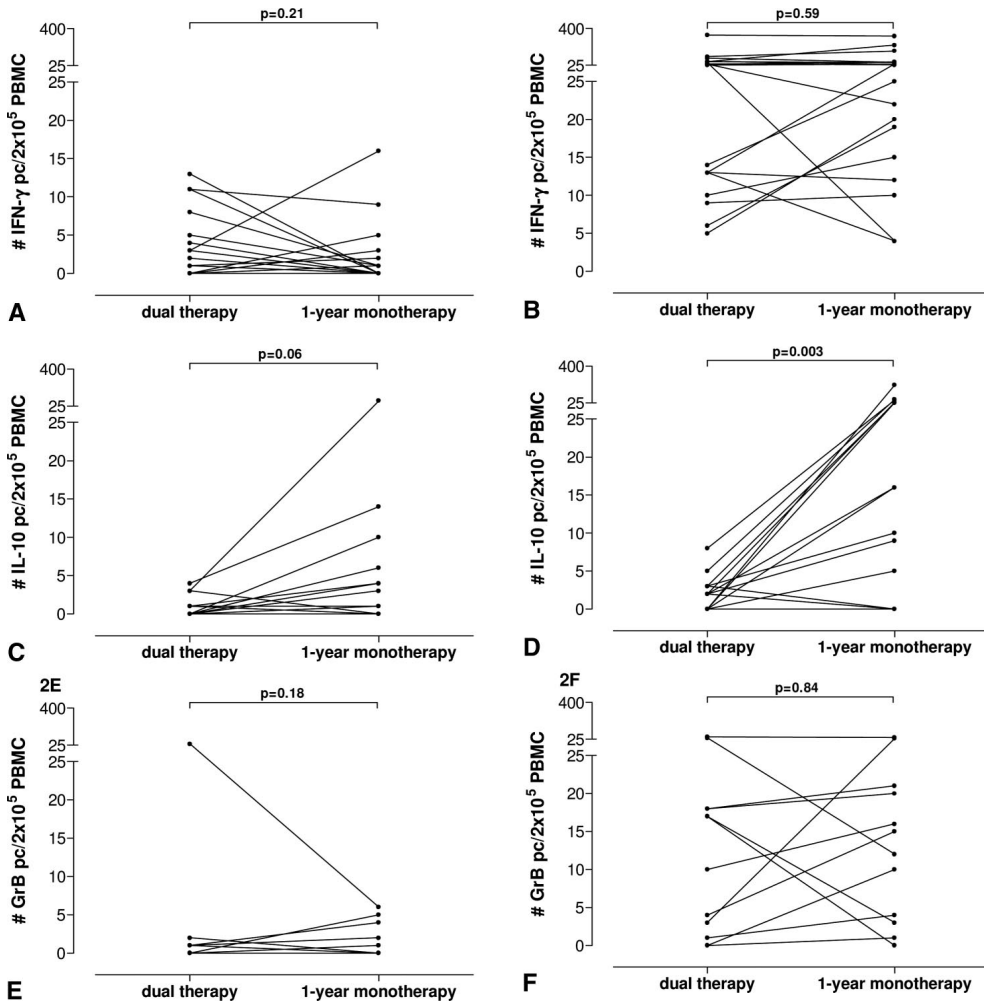
Figure 2: Number of IFN-γ, IL-10 and granzyme B (GrB) producing cells reactive to donor cells (A, C, E) and 3rd-party cells (B, D, F) before (dual therapy) and after (1-year monotherapy) discontinuation of AZA or MMF in PBMC from HLA-identical living-related renal transplants determined by IFN-γ, IL-10 and GrB Elispot assays.

Tetanus toxoid reactivity

The TET reactivity was not affected after withdrawal of AZA or MMF in IFN-γ Elispot assays [dual therapy: median, 6 IFN-γ producing cells/2×10^5 PBMC (0-217) vs. monotherapy: median, 4 IFN-γ producing cells/2×10^5 PBMC (0-179); p=0.74], and GrB Elispot assays [dual therapy: median, 5 GrB producing cells/2×10^5 PBMC (0-112) vs. monotherapy: median, 8 GrB producing cells/2×10^5 PBMC (0-115); p=0.44].

The total number of TET-reactive IL-10 producing cells was comparable during dual and monotherapy [dual therapy: median, 3 IL-10 producing cells/2×10^5 PBMC (0-77) vs. monotherapy: median, 10 IL-10 producing cells/2×10^5 PBMC (0-102); p=0.32].

However, the number of patients that could respond to TET antigens (≥5 cytokine producing cells/2x10^5 PBMC) in the IL-10 Elispot assay was significantly higher at monotherapy than at dual therapy (dual therapy, 5/20 (25%) vs. monotherapy, 13/18 (72%); p=0.008; Fisher's Exact test). No differences were found in IFN-γ (p=0.57) and GrB (p=0.43) Elispot assays.
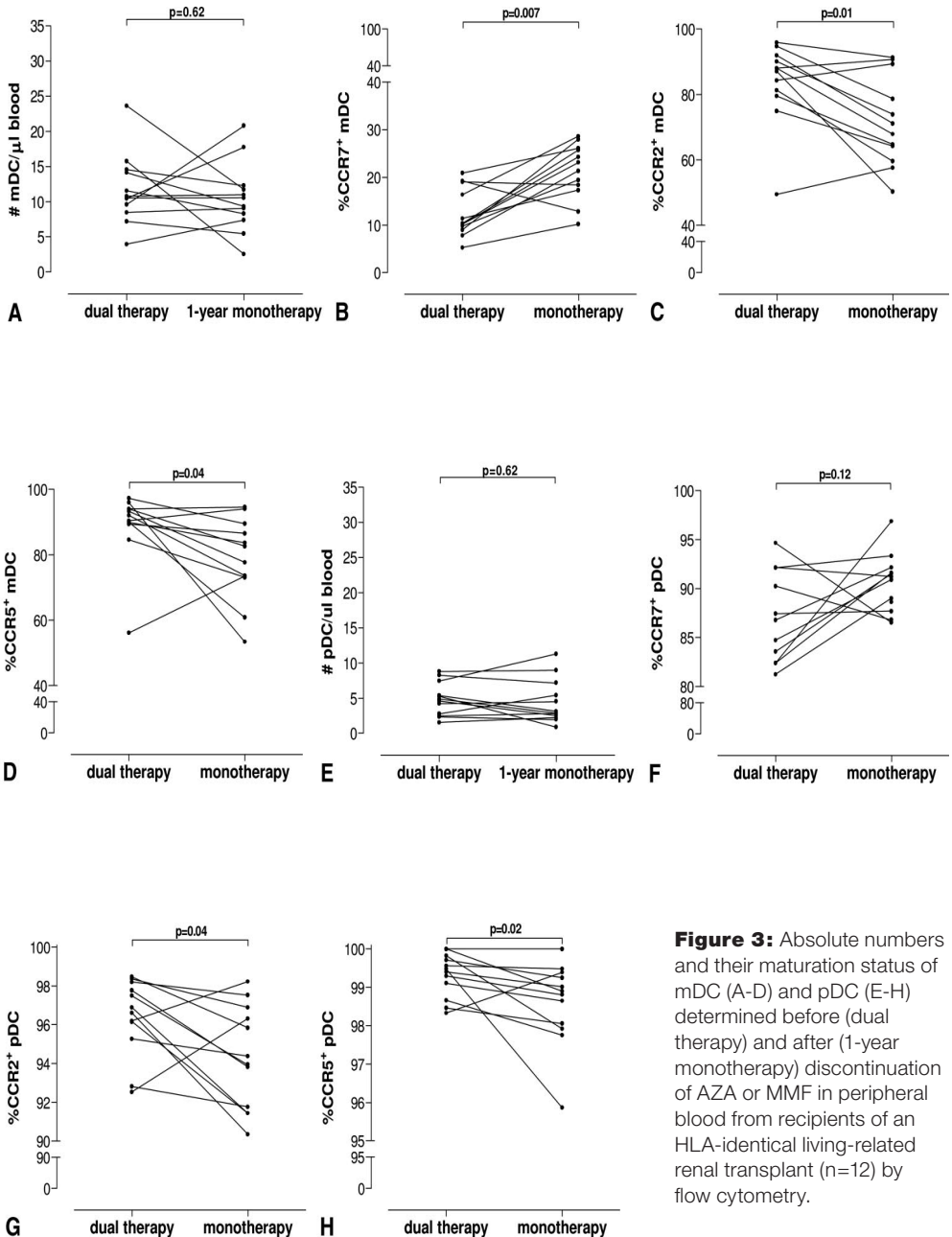

Figure 3: Absolute numbers and their maturation status of mDC (A-D) and pDC (E-H) determined before (dual therapy) and after (1-year monotherapy) discontinuation of AZA or MMF in peripheral blood from recipients of an HLA-identical living-related renal transplant (n=12) by flow cytometry.

Dendritic cell subsets and maturation status

Discontinuation of AZA or MMF had no effect on the total DC numbers [dual therapy: median, 14.6 DC/ml (range, 5.5-31.1); monotherapy: median, 14.4 DC/ml (3.5-26.2); p=0.52], the number of mDC [dual therapy: median, 10.7 ml mDC (4.0-23.7); mono-therapy: median, 10.0 ml mDC (2.6-20.8); p=0.62, Figure 3A] and pDC [dual therapy: median, 4.7 ml pDC (1.6-8.8); monotherapy; median, 3.0 ml pDC (0.93-11.3); p=0.62, Figure 3E], nor on the pDC/mDC ratio [dual therapy: median, 0.37 (0.23-0.82); monotherapy: median, 0.33 (0.16-0.96); p=0.49].

The percentage of CD83$^+$ mDC was not affected after discontinuation of AZA or MMF [dual therapy: median, 0.49% (0-2.8); monotherapy: median: 0.65% (0.08-1.45); p=0.47]. During monotherapy, the percentage of CCR7$^+$ mDC [median, 22.3% (10.2-28.6); p=0.007] was significantly higher than during dual therapy [median, 10.29% (5.29-20.96); Figure 3B]. The percentage CCR2$^+$ mDC [dual therapy: me-dian, 87.6% (49.5-95.9); monotherapy: median, 69.5% (50.3-91.4); p=0.01; Figure 3C] and CCR5$^+$ mDC [dual therapy: median, 91.2% (56.1-97.3); monotherapy: median, 80.1% (53.4-94.6); p=0.04; Figure 3D] was significantly lower during AZA or MMF.A similar pattern was found for pDC. No differences were observed in the percentage CD83$^+$ pDC [dual therapy: median, 0% (0-0.6); monotherapy: median, 0% (0-0.9); p=0.31] during dual and monotherapy. The percentage CCR7$^+$ pDC increased after discontinuation of AZA or MMF [dual therapy: median, 86.8% (81.3-94.7); monotherapy: median, 91.1% (86.6-96.9); p=0.12; Figure 3F]. The percentage CCR2$^+$ pDC [dual therapy: median, 96.8% (92.5-98.5); monotherapy: median, 94.2% (90.4-98.2); p=0.04; Figure 3G] and CCR5$^+$ pDC [dual therapy: median, 99.4% (98.3-100); monotherapy: median, 98.9% (95.9-100); p=0.02; Figure 3H] decreased after discontinuation of AZA or MMF.

Discussion

After solid organ transplantation, life-long use of immunosuppression is deemed necessary to prevent graft rejection. However, after HLA-identical LR renal trans-plantation, the necessity for long-term use of immunosuppression is yet unknown. Considering the severe side-effects of immunosuppression, minimizing of immuno-suppression in these transplant recipients might be beneficial. Theoretically, discon-tinuation of immunosuppression in organ transplant recipients might result into an in-creased donor-reactive T-cell response (12-14, 40). In this study, we showed that the number of donor, 3rd-party and TET-reactive IFN-γ producing cells or GrB producing cells did not increase after discontinuation of AZA or MMF. However, significantly in-creased numbers of 3rd-party (p=0.003) and TET-reactive (p=0.008) IL-10 producing cells and a trend in more donor-reactive IL-10 producing cells (p=0.06) were found in HLA-identical LR renal transplant recipients. Additionally, no acute rejections oc-curred (57). We suggest that the suppressive function of IL-10 was hindered by AZA

and MMF. AZA and MMF are anti-proliferative agents, and may also have an effect on cytokine production of lymphocytes (41-43).

IL-10 is an anti-inflammatory cytokine that have been associated with down regulation of the immune response (44). Several cells can secrete IL-10, such as B cells, mono-cytes, DC, activated Th2 cells, and regulatory T cells (44). In our assay, we assume that monocytes and DC did not produce IL-10, because only non-adherent cells were transferred to the IL-10 Elispot plate. Upon activation by donor cells, both Th2 cells and regulatory T cells mainly produce IL-10 (45). In the present study, we observed higher numbers of donor, 3rd-party and TET reactive IL-10 producing cells after with-drawal of AZA or MMF, while the number of IFN-γ and GrB producing cells remained stable. In agreement with our results, also other studies reported in HLA-identical LR renal transplant recipients the presence of donor-reactive IL-10 in HLA-identical LR renal transplant recipients that could reflect allograft tolerance (20, 46).
Renal transplant recipients who receive long-term immunosuppression are suscep-tible for infections (6). Therefore, tapering of immunosuppression may reduce the chance for infections in those patients. In our study, we observed that more patients could respond to TET antigens in IL-10 Elispot assays after discontinuation of AZA and MMF, suggesting an improvement of reactivity directed to nominal antigens.

Mismatches in mHAgs between donor and recipient in combination with the pres-ence of the correct HLA-restriction molecule may trigger T-cell reactivity (2). We analysed whether donor-reactive T-cell responses could be a result of known mHAg disparities between donor and recipient. In the present study, we again found no relation between mHAg mismatches and the number of donor-reactive cytokine producing cells, which is in agreement with our previous studies (9, 38). Further-more, Heinold et al. showed after cadaveric and living-related renal transplantation that mHAgs mismatches between donor and recipients had no significant effect on death-censored 5-year graft survival (5).

DC could play an important role in determining the balance between transplant toler-ance and immunity (47). It has been suggested that immunological monitoring of pe-ripheral blood DC subset numbers and their ratio might identify transplant recipients in whom the immunosuppressive load can be safely tapered (23). It is assumed that blood DC in healthy individuals display an immature phenotype and induce T cell un-responsiveness (48, 49). Immature DC are specialised in the capture of antigens, and transport them from peripheral tissues to secondary lymph nodes. Both donor and recipient DC could play a role in allograft rejection. Donor DC are transferred with the graft and can directly interact with recipient T-cells. Recipient DC in peripheral lymphoid organs can take up soluble donor antigens, infiltrate the graft, and present the antigens to T-cells (50). Little is known about circulating DC after withdrawal of

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immunosuppression in kidney transplant recipients. Several studies reported interference of immunosuppressive drugs with DC numbers, differentiation and maturation of DC (23, 29-31, 33). Our study showed that withdrawal of AZA or MMF had no effect on the absolute numbers of total DC, mDC and pDC and their ratio. Although, several problems related to monitoring DC phenotype and subsets in peripheral blood were reported, e.g. variability in technique, absence of reference standards, incomplete data regarding the influence of disease, medication, and patient-related factors on blood DC subsets (51). Additionally, some patients had mDC and pDC numbers that differed from the majority of patients. Nevertheless, interestingly, DC subsets shifted towards more mature DC phenotype after discontinuation of AZA or MMF, suggesting that AZA or MMF hinders the phenotypic maturation status of DC. In agreement with our data, it has been reported that MMF effects phenotypic DC maturation in both mice and in vitro models (52, 53). In MMF-treated DC a lower expression of CD40, CD80, CD86, CD83, and CD54 was observed, suggesting an inhibitory effect of MMF on DC maturation (52). Also a dose-dependent inhibition of MLR with AZA treated DC was reported (54). MMF is an inhibitor of the enzyme IMPDH, which is involved in the novo synthesis of guanosine nucleotides. The inhibitory effect of MMF on DC maturation could be caused by an imbalance between cyclic guanosine monophosphate and cyclic adenosine monophosphate (cAMP) in DC (55). Because both mDC and pDC circulate in peripheral blood (56), those data support our results that after discontinuation of AZA and MMF DC shifted towards a more mature phenotype.

In conclusion, recipients of an HLA-identical LR renal transplant can be safely withdrawn from AZA or MMF. The number of donor, 3rd-party and TET-reactive IL-10 producing cells and maturation of DC was suppressed by AZA and MMF, suggesting that these immunosuppressive drugs may hinder down regulation of the general immune reactivity, including allogeneic responses.

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Chapter 9

Summary and conclusions

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Summary

In this thesis we first describe the incidence of two important long term side effects, namely calcineurine inhibitor (CNI) induced nephrotoxicity and malignancies in a Dutch heart- and renal transplant population respectively, and its influence on patient- and graft survival. Knowing the negative influence of immunosuppression on these complications, we then explored the possibilities to reduce the immunosuppressive load long after transplantation in our HLA-mismatched- and a HLA-identical renal transplant population. Furthermore, we studied the influence of reducing the immunosuppressive load on clinical and immunological parameters.

Chapter 1 is a general introduction to the background and the aim of this thesis and describes three well known (long-term) complications after solid organ transplantation, i.e. cardiovascular disease, malignancy and CNI induced nephrotoxicity. The incidence and possible underlying mechanisms behind these complications after transplantation form the rationale to explore the possibilities of reducing the immunosuppressive load in a selected group of transplanted recipients.

In Chapter 2 we evaluated the development of end stage renal failure (ESRF) in heart transplant recipients after 21 years of heart transplantation in our clinic, and studied its influence on patient survival. Since an increased expression of the profibrotic transforming growth factor-ß (TGF-ß) is considered an important etiologic factor for CNI induced nephrotoxicity, we studied the impact of gene polymorphisms in codon 10 and 25 of the promoter region of TGF-ß on the risk of developing ESRF in these heart transplant recipients. In this observation period, the prevalence of ESRF after heart transplantation was high; almost one fifth of them had developed ESRF. Developing ESRF after heart transplantation results in very high mortality rates. We found a highly significant association between TGF- ß polymorphisms and CNI induced ESRF after heart transplantation. In the univariable Cox proportional hazards analysis we found a highly significant association between Pro carriers of either codon 10 or 25 and developing ESRF. In the multivariable analysis, the influence of TGF-ß codon 10 gene polymorphism remained significant. The implications of our findings are that maintenance immunosuppressive
regimens of cardiac allograft recipients with a TGF-ß codon 10 and probably also 25 gene polymorphism should no longer contain CNI, particularly because ESRF after heart transplantation results in an extremely high mortality rate.

Apart from chronic CNI induced nephrotoxicity, we have studied another long term complication after solid organ transplantation, namely the development of cancer. In all reports from different transplant centres all over the world, the incidence of almost all tumour types, skin and non-skin, is increased after solid organ transplantation compared with the general population.

In **Chapter 3** we described the incidence of cancer in the Dutch renal transplant population, transplanted between March 1966 and May 2008, and performed an analysis of patient and graft survival both from the day of transplantation and the diagnosis of cancer in recipients with invasive cancer in a nested case-controlled analysis. After 42 years of renal transplantation in the Netherlands, a significant proportion of patients have developed cancer. This feared complication of organ transplantation and treatment with immunosuppressive agents, comes at a significant later time point than other well known complications like, for example, infections and cardiovascular disease. Almost all patients who had developed cancer after transplantation die due to this complication, with a very short median survival after the diagnosis of cancer.
Our study shows that even compared with other renal transplant recipients, matched for gender, age, time after transplantation, and year of transplantation, developing cancer after renal transplantation significantly affects life expectancy and carries a poor prognosis with a limited patient survival after the diagnosis.

Tapering of immunosuppressive medication is indicated to prevent these long term side effects. In 2000, van Besouw et al had already shown that stable renal transplant recipients in our transplant population can be safely converted from calcineurin inhibitors to mycophenolate mofetil or azathioprine when their T-cell mediated donor reactivity, represented by donor-specific cytotoxic T-lymphocyte precursor frequencies (CTLpf), are low. In sequel to these results we wondered whether a low donor specific CTLpf had also predictive value for further reducing the immunosuppressive load in human leucocyte antigen (HLA) mismatched renal transplant recipients treated with duo therapy immunosuppression, consisting of prednisolone combined with mycophenolate mofetil (MMF) or azathioprine (AZA). In **chapter 4** we describe that gradually decrease the dose of AZA or MMF to 50% of the original dose at inclusion, is safe in these selected patients, without developing acute rejection or deterioration of renal function in the observation time.

In **chapter 5** the T-cell reactivity in these transplant patients after tapering of AZA or MMF is described. Tapering of the AZA or MMF dose resulted in a decrease of donor specific CTLpf in all patients with detectable donor specific CTLpf before reduction of their immunosuppression, while no effect on third-party reactive CTLpf were found. The T-cell reactivity to donor and third party cells as tested in mixed lymphocyte cul-

ture and in IFN-γ Elispot was not affected either by tapering of immunosuppression. It is concluded that reduction of immunosuppression results in a specific decrease of donor-directed cytotoxic capacity of immunocompetent cells, while their proliferation and cytokine production capacity remained unchanged. These results suggest that immunosuppression hinders development of cytotoxic non-responsiveness.

Chapter 6 describes a study of CNI withdrawal in stable renal transplant recipients, with donor-specific cytotoxic hyporesponsiveness, who were originally treated with a triple immunosuppressive regimen, consisting of the CNIs tacrolimus or cyclosporine and AZA or MMF combined with prednisone. Four months after CNI withdrawal, the mRNA expression of Granzyme B (GrB) and Perforin as markers for cytotoxicity, Fas and FasL as markers for apoptosis, T-bet and GATA3 as Th1 and Th2 transcription factors, and CD25 were significantly lower than during CNI treatment. After CNI discontinuation, donor-specific CTLpf decreased, while FOXP3 expression discriminated between detectable and non-detectable donor-specific cytolysis reactivity; FOXP3 transcript values were highest in absence of donor-specific cytotoxicity. It is discussed that, in kidney transplant patients indeed CNI interfere in the cascades leading to donor-specific hyporesponsiveness. In the absence of CNI, cytotoxic hyporesponsiveness was associated with high FOXP3 mRNA expression levels and suggests that donor-specific reactivity is the result of impaired regulation by FOXP3+ T-cells. After withdrawal of CNI, these regulatory mechanisms emerge.
Renal function, blood pressure and cholesterol were studied before and after CNI withdrawal at four months. In addition, these clinical parameters were analyzed in the CNI-free patients after further dosage reduction of AZA or MMF (two times 25%) at 24 months. No significant changes in renal function, blood pressure or serum cholesterol occurred after CNI withdrawal or dose reduction of AZA or MMF. These findings suggest that long-term treatment with CNI affects the mechanisms and cells in CNI-treated patients in such a way that damage occurred, and as a result no beneficial effect of CNI withdrawal on this clinical parameters can be found.

Although an immune respons to a renal allograft is mainly driven by HLA differences between the donor and the recipient, HLA-identical living-related transplant recipients are usually treated following the same immunosuppressive protocols as for non-identical pairs. Wondering whether this is correct, we conducted a study in stable HLA-identical living-related renal transplant recipients, in which the immunosuppressive medication is reduced dramatically. The results of this study are described in **chapter 7**. In our HLA-identical study group, dramatically tapering of their immunosuppressive medication to low-dose prednisolone monotherapy of 5 mg a day is well tolerated, without the occurrence of acute rejections during a follow up of 2 years. The role of tapering immunosuppression and the risk of recurrence of original disease is discussed. After studying our HLA-identical living-related renal transplant

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recipients under full dose immunosuppression and screening literature, we concluded that the prevalence of recurrence after tapering immunosuppressive medication in our study group was comparable with that described before in living-related HLA-identical renal transplant recipients who used full-dose immunosuppression and is in line with earlier observation.

Furthermore we questioned whether discontinuation of AZA or MMF influenced T-cell reactivity, determined by Elispot assays, in these HLA-identical patients. This Elispot assay determine the frequency of pro-inflammatory cytokine interferon-gamma (IFN-γ) and anti-inflammatory cytokine IL-10 that have been associated with allograft rejection or suppression of the immune response, respectively. Granzyme B (GrB) was used as a marker of activity of cytotoxic T-lymphocytes. Additionally, we wondered whether discontinuation of immunosuppression affected the circulating dendritic cell (DC) subsets numbers and their maturation status determined by flow cytometry, knowing that dendritic cells could play an important role in determining the balance between transplant tolerance and immunity. The results of this study are described in **chapter 8**. We showed that the number of donor, third-party, and tetanus (TET)-reactive IFN-γ producing cells or GrB-producing cells did not increase after discontinuation of AZA or MMF. However, significantly increased numbers of third-party and TET-reactive IL-10 producing cells and a trend in more donor-reactive IL-10 producing cells were found in HLA-identical LR renal transplant recipients, suggesting that the suppressive function of IL-10 was hindered by AZA and MMF. Withdrawal of AZA or MMF had no effect on the absolute numbers of total DC, myeloid DC and plasmacytoid DC or their ratio. Nevertheless, DC subsets shifted toward more mature DC phenotype after discontinuation of AZA or MMF, suggesting that AZA or MMF hinders the phenotypic maturation status of DC.

Conclusion and comment of recommendation

Long-term complications after transplantation are responsible for a decrease in patient and graft survival. This is illustrated by cardiovascular disease and malignancies being the first and second most frequent causes of death after transplantation and "death with a functioning graft" is still the most common cause of graft loss after solid organ transplantation. With the current patient and graft survival, the prevalence of these long-term complications is increasing and thus we have to explore the possibilities to reduce these problems in our transplant population. Causal factors like gender, race, age at transplantation, environment and lifestyle play an important role, but, on top of this, these long term complications are adversely affected by the use of a variety immunosuppressive drugs. Because, there are huge differences between various transplant centers in different countries concerning composition of population, acceptance criteria for recipients and donors and preferences for the immunosuppressive regimens, the complication rates are also different between individual centers and countries. So, results of different transplant centers as described in

literature can not be adopted indiscriminately by each individual transplant center or country and individual inventory of complications is indicated. In our retrospective analysis of the Dutch renal transplant population, we showed that cancer manifests rather late after transplantation and the prognosis is unfavorable. So, in prospective studies on this long term complication rate, patient follow-up time has to be at least ten years. The cause of the poor prognosis after being diagnosed with cancer after transplantation has to be annalysed in the near future. Although, long term studies on the complication rate of immunosuppression are difficult to perform and the results are influenced by many confounders these studies are indispensible.

Although all transplant recipients are at risk, there is an individual variable susceptibility for developing immunosuppression related complications, as we showed for TGF- ß gene polymorphism and CNI induced nephrotoxicity. On the other hand, we demonstrated that reduction of the immunosuppressive load after transplantation is possible in a selected group of patients and that long-term, high dose immunosuppression even seems to hinder the development of (partial) operational clinical tolerance. In the future, we have to focus on how we can identify patients who are at risk for developing these immunosuppression related complications and in whom the immunosuppression can be reduced without developing acute or chronic rejection of the graft. New techniques, like microarrays, make it possible to simultaneously screen lots of genes or proteins to identify new potential biomarkers for tolerance or rejection after transplantation. Prospective clinical trails are needed to assess the clinical relevance and usefulness of these biomarkers, so that finally the ultimate goal of tailor made immunosuppression for the individual patient can be reached.

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Meanwhile, based on the studies described in this thesis, we can make the following recommendations:

1. the immunosuppressive load after solid organ transplantation should be reduced, because it is associated with unwanted complications and hinders the development of tolerance
2. The use of CNI's in patients with a TGF- ß gene polymorphism of codon 10 leads to renal insufficiency and should therefore be avoided.
3. the use of triple immunosuppression in stable renal transplant recipients long after transplantation is not useful and can be safely reduced.
4. Immunological monitoring should be used in adapting immunosuppressive treatment
5. treatment of HLA-identical living-related kidney transplant patients with prednisone monotherapy 5 mg per day as maintenance immunosuppressive therapy is sufficient.

Chapter 10

Samenvatting en conclusie

Samenvatting en conclusies

In het eerste gedeelte van dit proefschrift beschrijven we de incidentie van twee belangrijke lange termijn complicaties na orgaantransplantatie, namelijk calcineurine geïnduceerde nefrotoxiciteit en maligniteiten, in respectievelijk een Nederlandse hart- en een niertransplantatiepopulatie en de invloed hiervan op patient- en graft survival. Aangezien het gebruik van immuunsuppressieve medicatie deze complicaties in de hand werkt, hebben we in het tweede gedeelte van dit proefschrift de mogelijkheden van afbouw van deze medicatie bestudeerd in een HLA gemismatchte en een HLA identieke niertransplantatiepopulatie. Daarbij hebben we de invloed van het afbouwen van immuunsuppressie bestudeerd op klinische en immunologische parameters.

Hoofdstuk 1 is een algemene inleiding over de achtergrond en het doel van dit proefschrift. Drie bekende en belangrijke lange termijn complicaties na orgaantransplantatie worden hierin beschreven, namelijk hart- en vaat ziekten, maligniteiten en calcineurine (CNI) geïnduceerde nefrotoxiciteit. De incidentie en de mogelijke onderliggende mechanismen van deze complicaties na orgaantransplantaie vormen de onderliggende reden om de mogelijkheden tot het verminderen van immuunsuppressie bij een vooraf geselecteerde groep transplantatiepatiënten te onderzoeken.

In **hoofdstuk 2**, beschrijven we het voorkomen van eind stadium nierfalen (ESRF) na hart transplantatie en bestuderen we de invloed van nierfalen op de patiëntoverleving. Aangezien een verhoogde expressie van de profibrotische transforming growth factor-β (TGF-β) als een belangrijke etiologische factor voor CNI geïnduceerde nefrotoxiciteit wordt beschouwd, bestudeerden we daarnaast de impact van gen-polymorfismen in codon 10 en 25 van de promotor regio van TGF-β op het risico van het ontwikkelen van ESRF in deze harttransplantatie ontvangers. De prevalentie van ESRF na harttransplantatie was in bovengenoemde observatieperiode hoog. Bijna een vijfde van de harttransplantatieontvangers had ESRF ontwikkeld. Het ontwikkelen van ESRF na harttransplantatie resulteert in zeer hoge mortaliteitcijfers. We vonden een sterk verband tussen TGF-β polymorfismen en CNI geïnduceerde ESRF na harttransplantatie. In de univariate Cox proportional hazards analyse vonden we een sterk significante relatie tussen Pro dragers van een codon 10 of 25 en het ontwikkelen van ESRF. In de multivariate analyse bleef alleen de relatie tussen TGF- genpolymorfisme van codon 10 en het risico op ESRF na harttransplantatie sterk significant aanwezig. De implicaties van onze bevindingen zijn dat de lange termijn immunosuppressieve regimes van harttransplantaatontvangers met een TGF-β codon 10 en waarschijnlijk ook 25 gen polymorfisme geen calcineurine remmers moeten bevatten, vooral omdat ESRF na harttransplantatie resulteert in een zeer hoog sterftecijfer.

Naast chronische CNI geïnduceerde nefrotoxiciteit, hebben we ook een andere lange termijn complicatie na een orgaantransplantatie onderzocht, namelijk de ontwikkeling van kanker. In alle rapportages vanuit verschillende transplantatie centra in de wereld, wordt er een verhoogde incidentie van bijna alle soorten kanker beschreven na orgaantransplantatie. Dit geldt zowel voor huid- als solide tumoren.

In **hoofdstuk 3** beschrijven we de incidentie van kanker in de Nederlandse niertransplantatiepopulatie, getransplanteerd tussen maart 1966 en mei 2008, en hebben we een analyse uitgevoerd van de patiënt- en transplantaatoverleving. Deze survival analyse is zowel uitgevoerd vanaf de dag van transplantatie als vanaf het moment dat bij een transplantatie patiënt de diagnose maligniteit gesteld is. Een gematchte populatie patiënten uit dezelfde transplantatieperiode zonder maligniteit vormde de controle groep. Na 42 jaar niertransplantatie in Nederland is duidelijk dat een aanzienlijk deel van de patiënten kanker ontwikkelt. Deze gevreesde complicatie van orgaantransplantatie ontstaat op een aanzienlijk later tijdstip dan de andere bekende complicaties zoals infecties en hart- en vaatziekten. Bijna alle patiënten die kanker hebben ontwikkeld na transplantatie overlijden ten gevolge van deze complicatie, met een zeer korte mediane overleving na de diagnose. Onze studie toont aan dat het ontwikkelen van een maligniteit na niertransplantatie de levensverwachting van deze patienten significant beinvloedt. Deze bevinding kwam uit de vergelijking met een controle groep bestaande uit niertransplatatiepatienten zonder maligniteit gematcht voor geslacht, leeftijd, tijd na de transplantatie, en het jaar van transplantatie.

Gezien de relatie tussen het gebruik van immuunsuppressie en het ontwikkelen van deze lange termijn complicaties, lijkt afbouwen van immuunsuppressieve medicatie na transplantatie geindiceerd. In het jaar 2000 hebben van Besouw et al reeds aangetoond dat, in stabiele niertransplantatieontvangers uit onze transplantatie populatie, de calcineurine remmers veilig konden worden veranderd in mycofenolaat mofetil (MMF) of azathioprine (AZA) indien de T-cel gemedieerde donor reactiviteit, gemeten m.b.v. de donor-specifieke cytotoxische T-lymfocyt voorloper frequentie (CTLpf), laag was. In vervolg op deze resultaten vroegen we ons af of een lage donor specifieke CTLpf ook een positief voorspellende waarde had voor een verdere verlaging van de immunosuppressieve belasting in stabiele, HLA-gemismatche niertransplantatiepatiënten die werden behandeld met dubbel therapie immunosuppressie, bestaande uit prednisolon in combinatie met MMF of AZA.

In **hoofdstuk 4** beschrijven we dat een geleidelijke afbouw van de dosis MMF of AZA tot 50% van de oorspronkelijke dosis bij inclusie veilig is bij deze geselecteerde patientengroep zonder dat acute afstoting van het transplantaat of een verslechtering van nierfunctie optrad in de observatietijd. In **hoofdstuk 5** wordt de T-cel reactiviteit bij deze patiënten na afbouwen van AZA of MMF beschreven. Geleidelijke vermindering van de AZA of MMF dosis resulteerde in een daling van donor specifieke CTLpf

bij alle patiënten met detecteerbare donor specifieke CTLpf vóór de reductie van hun immunosuppressie, terwijl er geen effect op volledig HLA-gemismatchte derde partij reactieve CTLpf werd gevonden. De T-cel reactiviteit tegen cellen van de donor en de derde partij, zoals getest in de gemengde lymfocyt cultuur (MLR) en IFN-γ Elispot, werd niet beïnvloed door de geleidelijke vermindering van immunosuppressie. Concluderend resulteerde de vermindering van immunosuppressie in een specifieke daling van de donor-gerichte cytotoxische capaciteit van immunocompetente cellen, terwijl hun proliferatie en cytokine productiecapaciteit ongewijzigd bleef. Deze resultaten suggereren dat immunosuppressie de ontwikkeling van cytotoxische non-responsiviteit belemmert.

Hoofdstuk 6 beschrijft een studie van CNI onttrekking in stabiele niertransplanta-tiepatiënten, met een lage donor-specifieke, cytotoxische respons, die oorspronkelijk werden behandeld met een drievoudige immunosuppressieve therapie, bestaande uit CNI's (cyclosporine of tacrolimus), AZA of MMF in combinatie met prednison. Vier maanden na CNI onttrekking, bleek de mRNA expressie van Granzym B (GRB) en Perforine als markers voor cytotoxiciteit, Fas en FasL als markers voor apoptose, T-bet en GATA3 als Th1 en Th2 transcriptiefactoren, en CD25 significant lager dan tijdens CNI behandeling. Na CNI onttrekking was de donor-specifieke CTLpf af-genomen, terwijl de Foxp3 mRNA expressie discrimineerde tussen detecteerbaar en niet-detecteerbare donor-specifieke cytolytische reactiviteit. De Foxp3 mRNA transcriptie was het hoogst in afwezigheid van donor-specifieke cytotoxiciteit. In de discussie wordt besproken dat CNI behandeling bij patiënten met een nier-transplantatie inderdaad interfereert in de cascades die leiden tot donor-specifieke hyporesponsieviteit. In afwezigheid van CNI was cytotoxische hyporesponsieviteit geassocieerd met een hoge Foxp3 mRNA expressie, wat suggereert dat donor-specifieke reactiviteit het resultaat is van een verminderde suppressie door Foxp3 + regulatoire T-cellen. Na het ontrekken van CNI's zie je deze regulatoire mechanismen weer ontstaan. Tevens werden, voor en 4 maanden na het onttrekken van CNI's, de nierfunctie, bloeddruk en serum cholesterol waarden van deze patienten vervolgd. Deze klinische parameters werden nog verder vervolgd in de CNI vrije patient na ver-dere dosisreductie van de AZA of MMF (tweemaal 25% dosisreductie) op tijdstip 24 maanden. Er hebben zich geen significante veranderingen voorgedaan in nierfunctie, bloeddruk of serum cholesterol waarden na CNI onttrekking of verdere dosisreductie van AZA of MMF. Deze bevindingen suggereren dat langdurige behandeling met CNI definitieve schade veroorzaakt en dat als gevolg daarvan geen gunstig effect van CNI onttrekking op deze klinische parameters werd gevonden.

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Hoewel een immuunreactie tegen een donornier voornamelijk bepaald wordt door HLA verschillen tussen de donor en de ontvanger, worden ontvangers van een HLA-identiek, living-related transplantaat meestal behandeld volgens dezelfde immunosuppressieve protocollen als voor niet-identieke koppels. Ons afvragende of dit correct is, hebben we een studie uitgevoerd met stabiele HLA-identieke, living-related niertransplantatiepatiënten, waarbij de immunosuppressieve medicatie drastisch werd verminderd. De resultaten van deze studie zijn beschreven in **hoofdstuk 7**. In onze HLA-identieke studiegroep werd het drastisch afbouwen van de immuunsuppressie tot een lage dosis prednisolon monotherapie van 5 mg per dag goed wordt verdragen zonder dat er acute afstoting optrad gedurende een follow-up van 2 jaar. De rol van het afbouwen van immunosuppressie en het risico op terugkeer van de oorspronkelijke ziekte in het transplantaat wordt besproken. Na het bestuderen van onze HLA-identieke, living-related niertransplantatiepatiënten onder volledige dosis immunosuppressie en screening van de literatuur, concluderen we dat de prevalentie van terugkeer van oorspronkelijke ziekte in het niertransplantaat na afbouw van de immunosuppressieve medicatie in onze studie groep, vergelijkbaar is met die eerder beschreven in living-related, HLA-identieke niertransplantatieontvangers behandeld met full-dose immunosuppressie en vergelijkbaar is met eerdere publicaties in de literatuur. Ook hebben we onderzocht of het staken van AZA of MMF in deze HLA-identieke patiënten de T-cel reactiviteit, gemeten middels een Elispot assay, beïnvloed. Deze Elispot assay bepaald de frequentie van het pro-inflammatoire cytokine interferon-gamma (IFN-) en het anti-inflammatoire cytokine IL-10 die, respectievelijk, geassocieerd zijn met rejectie van het transplantaat of onderdrukking van de immuunrespons. Granzym B (GrB) werd gebruikt als een marker van de activiteit van cytotoxische T-lymfocyten. Daarnaast vroegen we ons af of het stoppen van AZA of MMF in deze patiënten het aantal circulerende dendritische cel (DC) subsets en hun maturatie status, bepaald middels flowcytometrie, beïnvloedt, wetende dat dendritische cellen een belangrijke rol kunnen spelen bij het bepalen van het evenwicht tussen transplantatietolerantie en immuniteit. De resultaten van deze studie zijn beschreven in **hoofdstuk 8**. Hierin hebben we aangetoond dat het aantal donor-, derde-partij-, en tetanus (TET)-reactieve IFN- producerende cellen of GrB producerende cellen niet toenam na stopzetting van AZA of MMF. Er werd echter wel een significante toename van het aantal derde partij- en TET-reactieve, IL-10 producerende cellen en een trend in meer donor-reactieve, IL-10 producerende cellen gevonden. Dit suggereert dat de onderdrukkende werking van IL-10 werd gehinderd door AZA en MMF. Onttrekking van AZA of MMF had geen effect op de absolute aantallen van de totale DC, myeloïde- en plasmacytoïde DC's of hun verhouding. Niettemin, DC subsets verschoven in de richting van meer mature DC fenotype na het stoppen van AZA of MMF, wat suggereert dat AZA of MMF de fenotypische maturatie status van DC belemmert.

Conclusie en aanbevelingen

Lange termijn complicaties na transplantatie zijn verantwoordelijk voor een verminderde patiënt- en transplantaat overleving. Dit wordt geïllustreerd door het feit dat hart- en vaatziekten en kwaadaardige aandoeningen, respectievelijk de eerste en tweede meest voorkomende oorzaken van overlijden na de transplantatie zijn en dat 'overlijden met een functionerend transplantaat " nog steeds de meest voorkomende oorzaak van verlies van het transplantaat is na orgaantransplantatie. Met de huidige patiënt- en transplantaatoverleving, zal de prevalentie van deze complicaties in de toekomst verder stijgen. Daarom is het noodzakelijk om de mogelijkheden te onderzoeken om deze problemen in onze transplantatiepopulatie zoveel mogelijk te beperken. Oorzakelijke factoren, zoals geslacht, ras, leeftijd bij transplantatie, omgevingsfactoren en levensstijl spelen een belangrijke rol bij het ontwikkelen van deze complicaties, maar daarbij worden deze lange termijn complicaties ook in de hand gewerkt door het gebruik van een verscheidenheid aan immunosuppressieve geneesmiddelen. Doordat er enorme verschillen zijn tussen de diverse transplantatie centra in de wereld met betrekking tot de samenstelling van de bevolking, de acceptatie criteria voor ontvangers en donoren, en de voorkeuren voor immunosuppressieve behandelingsstrategieën, zijn de complicatie percentages eveneens verschillend tussen de afzonderlijke centra en landen. Hierdoor kunnen de resultaten van verschillende transplantatie centra, zoals beschreven in de literatuur, niet klakkeloos worden overgenomen door elk individueel transplantatiecentrum of land. Ieder centrum en/of land zal dus zijn eigen inventarisatie en evaluatie van de lange termijn complicaties na transplantatie moeten verrichten. In onze retrospectieve analyse van de Nederlandse niertransplantatiepopulatie, hebben we laten zien dat kanker zich vrij laat na transplantatie manifesteert en dat de prognose zeer ongunstig is. De follow-up periode in toekomstige prospectieve studies naar deze lange termijn complicaties zal dan ook minstens tien jaar moeten zijn. Verder onderzoek naar de achterliggende oorzaak van de slechte prognose na de diagnose kanker na transplantatie moet in de nabije toekomst plaatsvinden. Hoewel, lange termijn studies naar de complicaties van immunosuppressie bemoeilijkt wordt door deze noodzakelijke lange follow-up tijd en de resultaten beïnvloed worden door tal van confounders, zijn deze studies zijn onmisbaar voor onze transplantatiepatiënten.

Inmiddels kunnen we op grond van wat er in de studies die er in dit proefschrift beschreven staan al tot de volgende aanbevelingen komen:

1. de immuunsuppressieve druk na orgaan transplantatie zou i.h.a. moeten worden verminderd aangezien het gepaard gaat met ongewenste complicaties en het ontstaan van tolerantie hindert.
2. het gebruik van CNI's bij patienten met een TGF- genpolymorfisme van codon 10, leidt tot nierinsufficientie en zou dus moeten worden beperkt.

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3. drievoudige immuunsuppressie lang na nier transplantatie is niet zinvol en kan, in een stabiele patiëntengroep, veilig worden afgebouwd.
4. Immunologische monitoring zou moeten worden gebruikt bij het aanpassen van immunosuppressieve behandeling
5. HLA-identieke living-related niertransplantatiepatiënten hebben als onderhoud immuunsuppressive therapie voldoende aan 5 mg prednison monotherapie per dag.

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Abbreviations:

Arg	=	arginine
AZA	=	azathioprine
BCC	=	basal cell carcinoma
CNI	=	calcineurin inhibitor
CMP	=	cardiomyopathy
CMV	=	cytomegalovirus
CsA	=	cyclosporine A
CYP3A	=	Cytochrome P450, family 3, subfamily A
Dx	=	diagnosis
EBV	=	Epstein-Barr virus
ESKD	=	end stage kidney disease
ESRD	=	end stage renal disease
GFR	=	glomerular filtration rate
HLA	=	human leucocyte antigen
HTx	=	heart transplantation
IHD	=	ischemic heart disease
Leu	=	leucine
mHAgs	=	minor histocompatibility antigens
MMF	=	mycophenolate mofetil
NHL	=	non-Hodgkin's lymphoma
NMSC	=	non-melanoma skin cancer
NOTR	=	Netherlands Organ Transplant Registry
NTS	=	Netherlands Transplant Society
PCR	=	polymerase chain reaction
Pro	=	proline
PTLD	=	post-transplant lymphoproliferative disorders
PVD	=	primary valvular disease
RR	=	relative risk
RTx	=	renal transplantation
SCC	=	squamous cell carcinoma
SIR	=	standardized incidence ratio
Tac	=	tacrolimus
TET	=	tetanus toxoid
TGF-ß1	=	transforming growth factor- ß1

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Presentations   Year

- Oral   5th American Transplant Congress, Boston, USA   2004
- Oral   Nederlandse Nefrologie Dagen, Veldhoven, The Netherlands   2004
- Poster   37th Annual Meeting &Scientific Exposition of   2004
            American Society of Nephrology, St. Louis, USA
- Poster   XX international Congress of Transplant Society,   2004
            Vienna, Austria
- Poster   16e Bootcongres Nederlands Transplantatie Vereniging,   2004
            Texel, The Netherlands
- Oral   17e Bootcongres Nederlands Transplantatie Vereniging,   2005
            Maastricht, The Netherlands
- Poster   6th American Transplant Congress, Seatlle, USA   2005
- Oral   Nederlandse Nefrologie Dagen, Veldhoven, The Netherlands   2005
- Oral   26th Annual Meeting &Scientific Exposition of   2006
            The international Society for Heart and Lung Transplantation
            Madrid, Spain
- Invited   Symposium Farmacogenetica in de praktijk   2006
            Rotterdam, The Netherlands
- Oral   World Transplant Congress, Boston, USA   2006
- Oral   18e Bootcongres Nederlands Transplantatie Vereniging,   2006
            Zeewolde, The Netherlands
- Poster   13th ESOT Congress & 15th ETCO Congress,   2007
            Prague, Czech Republic
- Oral   7e Nederlandse Nefrologiedagen, Veldhoven, The Netherlands   2007
- Oral   7th American Transplant Congress , San Francisco, USA   2007
- Oral   Nederlandse Nefrologie Dagen, Veldhoven, The Netherlands   2007
- Oral   19e Bootcongres Nederlands Transplantatie Vereniging,   2007
            Zeewolde, The Netherlands
- Oral   20e Bootcongres Nederlands Transplantatie Vereniging,   2008
            Zeewolde, The Netherlands
- Invited   20 workshop Nefrologie, Nederlandse Federatie voor Nefrologie 2009
            Papendal. The Netherlands
- Oral   XXIII International Congress of The Transplantation Society   2010
            Vancouver, Canada
- Oral   22e Bootcongres Nederlands Transplantatie Vereniging,   2010
            Rotterdam, The Netherlands
- Oral   23e Bootcongres Nederlands Transplantatie Vereniging,   2011
Amsterdam, The Netherlands

Curriculum Vitae

Jacqueline werd geboren op 7 februari 1968 in Rotterdam. Zij groeide op in Rotterdam en deed daar in 1987 eindexamen V.W.O. aan de Thorbecke V.O. Na in eerste instantie uitgeloot te zijn voor de studie geneeskunde begon zij in 1987 aan de studie Biologie aan de Universiteit van Utrecht. Gelukkig kon zij twee maanden later alsnog Geneeskunde gaan studeren aan de Erasmus Universiteit te Rotterdam, waar in 1994 het artsexamen behaald werd. Na eerst twee jaar als AGNIO gewerkt te hebben in het Zuiderziekenhuis te Rotterdam, kon zij in 1996 daar beginnen aan de opleiding tot internist onder de leiding van Dr. A. Berghout. Op 1 januari 2000 werd de opleiding voortgezet in het Erasmus MC Rotterdam onder leiding van Prof. Dr. H.A.P. Pols en startte zij in 2002 met het aandachtsgebied Nefrologie onder begeleiding van Prof. Dr. R Zietse. Vanaf 2004 is zij werkzaam als Internist-Nefroloog in het Erasmus MC Rotterdam, alwaar het onderzoek dat heeft geleid tot het huidige proefschrift werd verricht.

In 2003 is zij, na 17,5 jaar samen, getrouwd met Laurens van Gelderen. Zij hebben twee prachtige zonen, Sven en Yven.

Dankwoord

Bedankt

Bedankt!
Als woord
Zo vaak gehoord

Bedankt!
Hoe vreemd
Een mens
Die het niet meent

Bedankt!
Voor alles
Voor iedereen

Bedankt!
't is echt
Het is oprecht

MIJN DANK!
Aan allen

Het is gezegd

Voor iedereen die het mogelijk heeft gemaakt dat dit proefschrift
en deze dag er gekomen is.

Rationale and effect
of reduction of
immunosuppressive load in
organ transplant recipients

Jacqueline van de Wetering