Chapter 1

Introduction and aims of the thesis
Introduction

Chronic kidney failure and renal transplantation

Kidney transplantation currently represents the treatment of choice for most patients with end-stage renal failure. Worldwide, the population treated with renal replacement therapy reached almost 1.7 million at the end of 2003, representing approximately 1.3 million patients who undergo dialysis and 400,000 patients who are alive with a kidney transplant. The number of patients treated for end-stage renal disease has doubled during the last decade in the United States and Europe, where dialysis consumes about 2% of health care budgets. Based on the European Dialysis and Transplant Association registry of the European Renal Association (ERA-EDTA), about 550,000 European patients are currently undergoing renal replacement therapy. Approximately 39% of these patients are on dialysis and 46.2% of them are living with a functioning graft. The percentage of kidneys transplanted from living donors varies widely among the European countries, ranging between less than 10% up to more than 50% (ERA-EDTA Registry: ERA-EDTA 2010, Annual report). The exponential increase in the number of patients on the waiting list for a renal transplant has globally become a serious problem fueled by an increase in the number of patients with end-stage renal disease, the shortage of donor organs, and the failure of transplanted kidneys. Renal graft can be lost shortly (within months) after transplantation due to acute rejection, whereas the major cause of long-term graft loss is the chronic transplant dysfunction.

Mechanism of acute transplant rejection

Acute rejection is a cell-mediated immune response of the recipient against the alloantigens present on the graft. During the past 10 years, crucial pathways involved in the T cell activation, triggering the acute rejection, have been elucidated. Naïve T cells require two signals to become fully activated. First, there is an interaction between recipient T cell receptor (TCR) on the one hand, and the antigenic peptide presented on the major histocompatibility complex (MHC) of the “professional” antigen presenting cells (APC) and other alloantigens exposed on the “non-professional” APC (such as residential tubular epithelial, mesangial and endothelial cells), on the other hand. The second signal comprises the co-stimulatory interaction between CD28 on T cells and its ligands CD80 (B7-1) and CD86 (B7-2) on APC. The CD28-B7 interaction induces activation and proliferation of T lymphocytes. Pro-inflammatory cytokines and chemokines are released and activate cytotoxic T cells, B cells, phagocytes and natural killer cells, which eventually lead to destruction of the graft. The molecular mechanism of acute rejection is summarized...
in Figure 1. The alloimmune response will also trigger the formation of regulatory T cells (Treg), mostly CD4+CD25+foxp3 subpopulation, which are able to steer the immune reaction towards tolerance. However, spontaneous activation of this counter-balance mechanism is normally insufficient for cessation of the allograft rejection.

With the current immunosuppressive therapy, acute rejection is not a primary cause of the graft loss anymore. Nevertheless, the immunosuppressive therapy is accompanied by serious side-effects. Moreover the occurrence and the number of acute rejection episodes are the most important risk factors for the development of the chronic transplant dysfunction.

Figure 1. Scheme depicting naïve T cell activation leading to acute rejection. APC-antigen presenting cells, MHC-major histocompatibility complex, TCR-T cell receptor, CTLs-cytotoxic T lymphocytes, and NK-natural killer cells (modified from Deng Sh. et al; Molecular Medicine Today; 1999)
Mechanism of chronic transplant dysfunction (CTD)

Despite the large improvement in the short-term renal allograft survival, long-term survival did not change considerably over the past 20 years. One of the reasons for this lack of improvement is that the currently available immunosuppressive therapy does not prevent CTD, which is the leading cause of graft loss at 1 year after transplantation\textsuperscript{10,11}. CTD is highly prevalent, with moderate to severe CTD present in 24.7\% of recipients at 1 year post-transplantation and in 90\% of recipients at 10 years after transplantation\textsuperscript{12}. Although patients can resume dialysis after graft failure, the loss of the transplanted kidney increases the risk of death threefold, substantially decreases the quality of life and increases the healthcare costs\textsuperscript{13,14}.

CTD is characterized by a relatively slow, but progressive loss of renal function which can start as soon as 3 months after transplantation. This coincides with chronic histopathological lesions such as transplant vasculopathy, interstitial fibrosis, tubular atrophy and focal glomerulosclerosis\textsuperscript{11,15}. Even in those transplants that are well functioning during the first months after surgery, a progressive decline in graft function develops in time, associated with proteinuria, \textit{de novo} or worsening of pre-existing hypertension and progressive tissue damage\textsuperscript{15}. Unlike in acute rejection, both non-immune and immune factors are implicated in the pathogenesis of CTD. Amongst the non-immune factors, the ischemia-reperfusion time of the graft during transplantation as well as the deleterious effects of calcineurin inhibitors on endothelial function are well known contributors to CTD\textsuperscript{11}. Immune factors include the histocompatibility differences between the donor and the host, chronic immune stimulation of donor-derived endothelium and the appearance of allospecific antibodies\textsuperscript{5}. A key role in the progression of renal damage to end-stage renal failure is played by the progressive loss of renal mass secondary to immune damage, hypertension and proteinuria, whereby the graft enters in a circle of self-perpetuated injury\textsuperscript{16}. Several risk factors have been implicated in the genesis/progression of CTD. The most important risk factor for development of CTD is the occurrence (and the number) of acute rejection episodes\textsuperscript{15}. Recipients who have had repeated acute rejection episodes show a lower graft survival rate than those with none or only one acute rejection episode\textsuperscript{17,18}. Moreover, the estimated half-life for cadaveric grafts is considerably shorter for the grafts that survived acute rejection than for those that did not have an acute rejection, i.e. 6.6 years vs 12.5 years\textsuperscript{19}. HLA mismatching constitutes another risk factor for CTD, as estimated half-life of graft survival decreased from 12.4 years in patients that are fully matched to 8.6 years for those with HLA-mismatched grafts\textsuperscript{20}. Further, recipients sensitization (anti-HLA antibodies elicited by pregnancies, blood transfusion or failed
transplants)\textsuperscript{21}, age and race\textsuperscript{15} and, unfortunately, inadequate immunosuppression constitute additional risk factors for CTD\textsuperscript{22,23}. Graft survival is further limited by factors that less obviously relate to rejection, such as donor age\textsuperscript{24,25} and source (living vs cadaveric)\textsuperscript{26,27}, hypertension\textsuperscript{28}, proteinuria\textsuperscript{15}, hyperlipidemia\textsuperscript{29} and smoking\textsuperscript{30}.

Despite several attempts to slow-down the progression of CTD, and because of the incomplete understanding of the multiple mechanisms underlying the CTD, there is no efficient strategy for its prevention or cure available so far. Hence, this is the main and most challenging problem in the field of renal transplantation. The prevention of acute rejection and the avoidance of the chronic use of systemic immunosuppressive therapy (either by local immunosuppression or by induction of tolerance) likely represent the principle measures to attenuate CTD.

\textit{Systemic immunosuppressive therapy}

Patients who have undergone transplantation must receive lifelong immunosuppressive therapy even if the donor and the recipient are completely matched for the major histocompatibility complex (HLA)\textsuperscript{5}. There is a possible relationship between the minor histocompatibility antigens and the alloimmune reaction\textsuperscript{31}, underlying the fact that only matching for HLA is not sufficient. Immunosuppressive treatment is particularly successful in limiting acute rejection episodes, but does not considerably increase the long-term graft survival. Moreover, chronic immunosuppressive treatment is accompanied by serious and potentially life-threatening side-effects. Thus, the effectiveness of renal transplantation is limited by the problems caused by immune incompatibility between graft and host\textsuperscript{32}.

Since the early days of kidney transplantation in the 1960s, the incidence of acute rejection has been reduced from over 80\% to below 15\% at the beginning of the twenty-first century. This is mostly due to standardized use of immunosuppressive therapy. During this period, the introduction of calcineurin inhibitors (CNIs) and the use of corticosteroids has led to a doubling of 1-year graft survival rate from around 45\% to 90\%\textsuperscript{11}. Despite their effectiveness, the currently available immunosuppressive treatments also have serious drawbacks. Intrinsically, because of non-selective suppression of the immune system, immunosuppressive treatments mitigate also the immune response against bacteria, fungi, tumor cells and viruses. As a result, patients frequently suffer from serious side-effects, such as opportunistic infections\textsuperscript{33} and malignancies\textsuperscript{34}. In addition, traditional immunosuppressive therapy negatively influences several cardiovascular risk factors, and significantly contributes to the development of cardiovascular co-morbidity\textsuperscript{35}. Furthermore,
CNIs are nephrotoxic and may therefore contribute to structural damage of the graft. Nowadays attempts are being made to decrease the incidence of these side-effects by limitation of the corticosteroids and minimization or complete withdrawal of CNIs. However, these measures can only be implemented in patients with low risk for rejection.

Thus, immunosuppressive drugs prolong the graft survival and improve the short-term success of organ transplantation, but impair the systemic immunity with increased risk of malignancy and infection and, though drug-specific side effects, they increase the risk of metabolic and cardiovascular co-morbidity and may even contribute to the development of CTD.

Given the drawbacks of the current immunosuppressive treatment, the improvement of transplantation outcome is critically dependent on development of novel therapeutical strategies. In that respect, development of a local rather than generalized immunosuppressive therapy may improve both graft survival and quality of life of the transplanted patients. Such a local treatment may selectively inhibit the alloantigen immune response to the donor graft in the absence of (or dramatically reduced) systemic immunosuppression in the recipient. The Holy Grail of transplantation immunopharmacotherapy is induction of graft-specific tolerance. Also in this respect, a local strategy of interference with specific molecular pathways involved in the immune cell (in)activation in the renal graft may constitute an appealing approach.

**Local gene therapy as an alternative for systemic immunosuppression**

One of the most attractive approaches for local immunosuppressive treatment relies on gene therapy. Gene therapy is a form of molecular treatment based on the delivery of exogenous nucleic acids into target cells, using various vectors. Originally used as a strategy to replace altered or missing genes in inherited diseases, gene therapy has later also been used to treat acquired pathologic conditions. Amongst these, gene delivery has been used in experimental organ transplantation as a tool to induce local immune suppression or to deliver cell-protective molecules. Renal transplantation is an ideal condition for gene therapy, as there is an opportunity to perform *ex vivo* genetic manipulation of the graft during the surgery itself. Selective and/or peripheral inhibition of allogeneic immune reaction at the local organ level through gene therapy can be accomplished by: (1) targeting the interaction between antigen-presenting cells (APC) and T cells; (2) limiting cytokine expression by already activated T cells and (3) inhibition of the pathway leading to cytotoxic T cell and B cell proliferation. Ideally, more than one pathway should be
modified by one single gene. An appealing candidate for gene therapy in this context is the enzyme Indoleamine 2.3-dioxygenase, which shall be further discussed in this thesis.

So far, several studies using both local and systemic administration and employing different vector systems demonstrated the beneficial effect of gene therapy on experimental acute renal rejection\textsuperscript{42,43}. Our research group introduced a successful renal targeting strategy using an RGD-modified first generation type 5 adenovirus\textsuperscript{44}. Local gene therapy with RGD-adenovirus delivered-IL-13 attenuated the acute rejection in a fully mismatched rat model of kidney transplantation, to the same extent as the systemic therapy (intramuscular injection of the vector) did in the same model. Importantly, in that study, no additional immunosuppressive therapy was used\textsuperscript{45}.

Nevertheless, the data describing the use of gene delivery systems for therapy of CTD are scarce. The prevention of CTD may require long-lasting expression of the therapeutic transgene, whereas the first generation adenovirus employed by us allows gene expression for a limited period of time (1-2 weeks)\textsuperscript{46}. However, under the influence of the immunosuppressive effect of the transgene, the viral vector may persist longer in the renal graft\textsuperscript{47}. Furthermore, the use of a target molecule they may induce alloantigen specific tolerance in “one hit” is another possible strategy to cope with the short-term expression of the transgene delivered by our adenoviral vector. Alternatively, donor-derived dendritic cells may be used as “carriers” to target the molecule of interest at the site of the alloimmune reaction. Both these strategies will be approached in this thesis.

The ultimate solution to avoid life-long systemic immunosuppressive therapy and its associated side-effects is the induction of permanent donor-specific tolerance, i.e. the lack of the recipient’s immune response to the donor graft in the absence of systemic immunosuppression and of immunodeficiency of the recipient\textsuperscript{48}. Over the past 50 years, rodent models have served as a tool to elucidate the mechanisms of tolerance to alloantigens. Also, several tolerogenic strategies have been proved successful in such models\textsuperscript{49}. However, in humans, induction of tolerance has only been reported in selected patients after the administration of aggressive conditioning regimens\textsuperscript{50,51}. Cessation of immunosuppressive medication leads usually to graft rejection, with less than 1% of kidney transplant patients showing (“operational”) tolerance\textsuperscript{52}. Studies in these later patients documented that transplant tolerance is associated with the presence of a regulatory phenotype, such as high frequency of CD4+CD25+foxp3\textsuperscript{+} T cells and high levels of IL-10\textsuperscript{53,54}. Like natural tolerance, transplantation tolerance can be achieved by controlling the reactivity of T cells by two mechanisms. The first mechanism, central (thymic) tolerance involves deletion of T cells recognizing self-antigens and selection of those directed against
nonsel antigens. The second mechanism, peripheral tolerance, involves the anergy (functional inactivity) of T cells, induction of suppressive, regulatory T cells, peripheral deletion (apoptosis) of alloreactive T cell or ignorance (lack of response to alloantigens). Indoleamine 2.3-dioxygenase

Indoleamine 2.3-dioxygenase (IDO) is an appealing candidate to provide protection from both acute and chronic rejection following organ transplantation. IDO is a 45 kD, heme-containing enzyme which catalyzes the first and rate-limiting step in the breakdown of the essential amino acid L-tryptophan along the kynurenine pathway (Figure 2). IDO expression is most potently activated by INF-γ, however it can also be induced by other inflammatory cytokines such as IFN-α, IFN-β, TNF-α, TLR-ligands, GITR ligands (glucocorticoid-induced tumor necrosis factor receptor ligands) and HDACs (histone deacetylases). IDO is expressed in a variety of cell types including placental cells, smooth muscle cells, fibroblasts, macrophages, endothelial cells and tumor cells, as well as in organs with large areas of mucosal tissue such as lung and gut.

Historically, IDO has been known as an enzyme involved in the host defense against infections. IDO induction by INF-γ in Toxoplasma gondii-infected fibroblasts was shown to limit intracellular parasite growth, due to the tryptophan deprivation in cell culture medium (later called “tryptophan starvation theory”). In 1998, Munn and Mellor opened up a new era for the IDO research, by documenting IDO expression in the placenta as a crucial factor involved in the foetal-maternal tolerance. This study set the stage for the use of IDO as a therapeutic target in several immune-mediated diseases as well as in the transplant-related pathology.

The mechanisms behind the immunosuppressive or tolerogenic effects of IDO are complex and still incompletely understood. Briefly, three mechanisms of action of IDO are considered. The first one is the tryptophan depletion, as the tryptophan is necessary for cell growth. The second mechanism is the generation of cytotoxic metabolites-mainly kynurenine, 3-OH-kynurenine and 3-OH-antranilic acid, which exert direct toxic effects on T cells (cell cycle arrest and apoptosis). Thirdly, IDO promotes differentiation of Tregs from naïve CD4 T cells and thereby induces tolerance. However, these three theories are not mutually exclusive; low tryptophan concentration enhances the immunosuppressive effect of some kynurenine metabolites, down regulates T cell receptor zeta-chain and induces a regulatory phenotype of naïve T cells.
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Furthermore, IDO is expressed by dendritic cells (DC) under certain conditions. The IDO+DCs are able to either convert naïve CD4 T cells into Tregs or activate mature Tregs to achieve full suppressor function. However, it is not fully clarified whether generation of Tregs induce IDO+DC or whether generation of Tregs follows IDO expression in DC. Moreover, the ability of DC to produce IDO does not seem to be equally distributed among the various subsets.

Figure 2. Tryptophan metabolism along the kynurenine pathway. Schematic representation of tryptophan breakdown mediated by IDO. Immunologically active IDO metabolites are highlighted in the boxes.
Until now, several transplantation studies using IDO as a treatment of (mainly) acute rejection have been reported, from a range of mouse to rat models. Genetic overexpression of IDO by different vectors was shown to prolong corneal\(^{66}\), cardiac\(^{67}\), islets\(^{68}\) and lung\(^{69}\) allograft survival.

The literature on IDO and kidney transplantation is limited. In a mouse model of spontaneous kidney allograft acceptance evidence for an IDO-regulatory dendritic cell mechanism of late acceptance was reported, whereas early acceptance seemed to be mediated by transforming growth factor (TGF)-beta and regulatory T cells\(^{70}\). In human kidneys with acute rejection, the expression of IDO has been reported\(^{71}\); however, the exact role of IDO in this context is unclear. In addition, the therapeutic potential of IDO in both acute rejection and chronic transplant failure after renal transplantation has not yet been explored.

**Aim of the thesis**

The aim of this thesis is to investigate the therapeutic potential of IDO in kidney transplantation. To this end, we study the effects of gene therapy with IDO, in both *in vivo* and *in vitro* experiments. As vector for gene delivery, we use our well-established RGD-modified adenovirus. Moreover, we investigate the expression of IDO in patients after renal transplantation. In **chapter 2** we investigate the effects of local gene therapy with adenovirus-delivered IDO in a rat model of acute rejection. In **chapter 3** we use the same approach to analyze the effects of IDO gene therapy in a rat model of chronic transplant dysfunction. In **chapter 4** we study *in vitro* the effects of genetically modified dendritic cells overexpressing IDO on the proliferation and the phenotype of T cells. In **chapter 5** we examine the IDO expression in renal human biopsies and we analyze tryptophan and kynurenines levels in both serum and urine of patients who underwent renal transplantation. **Chapter 6** gives a summary of the current thesis, discusses possible mechanisms of IDO effects and outlines future perspectives of gene therapy with IDO. Moreover, it describes a newly-identified antifibrotic effect of IDO.
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