The therapeutic potential of indoleamine 2.3-dioxygenase in kidney transplantation

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Chapter 3

Local gene therapy with adenovirus-delivered Indoleamine 2.3-dioxygenase ameliorates chronic kidney transplant dysfunction and promotes tolerance


submitted
Abstract

**Background:** Chronic transplant dysfunction (CTD) is the primary cause of late allograft loss in kidney transplantation and remains the major challenge in the field of kidney transplantation. Indoleamine 2,3-dioxygenase (IDO) is crucially involved in foeto-maternal tolerance and inhibits acute allograft rejection following kidney transplantation. The aim of the current study is to investigate whether intra-graft gene therapy with IDO is able to attenuate CTD.

**Methods:** Kidney transplantation was performed in a rat Dark-Agouti to Wistar-Furth CTD model. During the transplantation procedure, donor kidneys were incubated with either a modified adenovirus carrying the IDO gene (RGD-AdTIDO, n=7), a control adenovirus (RGD-AdTL, n=5) or saline (n=5). Recipients received low-dose cyclosporine during the first 10 days post-transplantation. Body weight, blood pressure, serum creatinine and proteinuria were measured every two weeks. Rats were sacrificed after 12 weeks and kidneys were analyzed using (immuno) histochemistry and real time PCR.

**Results:** Plasma creatinine levels and creatinine clearance were slightly increased by the end of the study and did not significantly differ between groups. RGD-AdTIDO significantly improved body weight gain and reduced systolic blood pressure compared to controls. Additionally, IDO therapy significantly decreased proteinuria, the transplant vasculopathy and the number of graft-infiltrating macrophages at week 12. The mRNA levels of foxp3 and TGF-β were elevated in the IDO-treated group as compared to the saline group.

**Conclusion:** Here we show for first time the beneficial effect of local IDO gene therapy on CTD in a rat model of renal transplantation.
**Introduction**

Although the rate of acute graft loss has substantially been reduced during the last decades due to novel (and combination of) immunosuppressive medication together with better organ preservation techniques, long-term graft survival has not improved considerably\(^1\)\(^2\). Consequently, the main cause of graft loss is the chronic transplant dysfunction (CTD)\(^2\).

CTD is characterized by a progressive loss of renal function, proteinuria and de novo or worsening of pre-existing hypertension, coinciding with chronic histopathological lesions such as transplant vasculopathy, interstitial fibrosis, tubular atrophy and focal glomerulosclerosis\(^3\)\(^-\)\(^5\). The development of CTD is a multifactorial process including both immune and nonimmune factors\(^6\)\(^-\)\(^7\).

Several approaches have been employed to reduce the occurrence of CTD, including the use of living donors, reduction of the incidence of acute rejection episodes, shortening of cold-ischemia time, the use of machine perfused instead of cold-stored renal grafts, and optimization of chronic immunosuppressive medication\(^8\)\(^-\)\(^10\). Despite these efforts, there is still no prevention strategy nor an effective treatment available for CTD. Therefore, the improvement of long-term graft survival remains the major challenge in the field of renal transplantation and the search for new targets of intervention in CTD is still ongoing. Indoleamine 2,3 dioxygenase is the rate-limiting enzyme in the metabolism of tryptophan, whereby the essential amino acid L-tryptophan is converted into its catabolites, the kynurenines\(^11\). It has recently been proposed that IDO has profound immunoregulatory activity and the concept that cells expressing IDO can suppress T-cell response and promote tolerance is a relatively new paradigm in immunology\(^12\). Considerable evidence now supports this hypothesis, including studies regarding mammalian pregnancy, tumor resistance, chronic infections and autoimmune diseases\(^13\)\(^-\)\(^16\). In organ transplantation, several studies have indeed confirmed the potential of IDO gene therapy in preventing acute rejection of skin, lungs, heart and pancreatic islets\(^17\)\(^-\)\(^20\). In addition, we recently demonstrated the protective effect of adenoviral gene therapy with IDO against acute rejection following kidney transplantation in rats\(^21\).

The evidence about the role of IDO and/or its therapeutic effect in chronic transplant dysfunction is limited to one study showing that sleeping beauty-based gene therapy with IDO inhibits lung transplantation-associated chronic complications\(^22\). Here we employed adenovirus-delivered IDO gene therapy to study the effects of IDO in a rat model of renal CTD.
Material and Methods

Experimental design

Experiments were performed in a rat kidney transplant model for CTD, in which inbred male Dark Agouti rats (DA/OlaHsd, Haplotype RT1<sup>av1</sup>) were used as donors and male Wistar Furth (WF/NHsd, Haplotype RT1<sup>u</sup>) rats were used as recipients. Body weight of the animals at the time of the transplantation was 210-250 g. The animals were housed in a light- and temperature-controlled environment and fed standard rodent chow and water ad libitum. Experimental protocols were approved by the Animal Research Ethics Committee of the University of Groningen, The Netherlands.

Adenovirus-mediated gene delivery in donor kidneys followed by orthotopic kidney transplantation was performed as previously described<sup>23</sup>. Briefly, donor kidneys were infused with adenovirus solution via the renal artery and incubated for 20 min in cold (4ºC) saline. After perfusion with saline, kidneys were transplanted by an end-to-end anastomosis of the renal artery, vein and ureter. Warm ischemia-time was 25 min. To prevent acute rejection, recipients received low dose cyclosporine A (CsA; 5 mg/kg body weight; Sandimmune, Novartis, Arnhem, The Netherlands) subcutaneously during the first 10 days post transplantation. The native kidney was removed after 10 days.

To investigate the effects of IDO gene therapy in this model, three groups of animals were included. In a first group, donor kidneys were infused with an RGD-adenovirus carrying the reporter gene GFP and the gene for human IDO (RGD-AdTIDO group). In a second group, an adenovirus carrying the reporter genes GFP and luciferase was used (RGD-AdTL group). An additional group receiving saline was included as control (saline group). Seven animals (3 from RGD-AdTL group, 1 from RGD-AdTIDO group and 3 from saline group) in which surgical and urological complications were observed were terminated at nephrectomy (day 10). The follow-up was 12 weeks. Blood pressure was measured every 2 weeks. Also, blood samples and 24 hrs urine were collected every 2 weeks. At the end of the study the rats were anesthetized with isoflurane and an aortic blood sample was collected. Plasma was isolated and stored at -80ºC. The kidney was perfused with saline and removed. A midcoronal slice was fixed in 4% paraformaldehyde, processed for paraffin embedding and further used for (immuno)histochemistry. A second slice was snap-frozen in liquid nitrogen, stored at 80ºC and further used for mRNA isolation.

Adenoviral vectors

RGD-modified adenoviruses<sup>24</sup> were used as vectors for gene delivery. A first generation recombinant adenovirus type 5 having an RGD sequence in the HI loop was kindly
provided by Dr. David T. Curiel (University of Alabama at Birmingham, Birmingham, Alabama, USA). This adenovirus contains the genes for green fluorescent protein (GFP, T) and firefly luciferase (L) under the control of a CMV promoter, in the E1 region (RGD-AdTL). To construct the IDO vector, the IDO gene was isolated from human placenta using PCR. Next, the cDNA of human IDO gene (hIDO) was cloned into the shuttle-plasmid pAdTrack-CMV\textsuperscript{25}. Using homologous recombination in *Escherichia coli* BJ5183, the shuttle was integrated into the RGD adenovirus plasmid pVK503\textsuperscript{26}, resulting in a RGD modified adenovirus genome with GFP and hIDO under the control of a CMV promoter cloned into the E1 region. Viruses were propagated on HEK 293 cells and purified by double CsCl density centrifugation. The amount of viral particles (VP) was determined spectrophotometrically at 260 nm. The infectivity of the viruses was determined by plaque assay on HEK 293 cells and expressed as plaque forming units per milliliter of virus stock (pfu/ml). The VP/pfu ratio of the virus stocks was 100:1 for both RGD-AdTL and RGD-AdTIDO.

**HPLC assay for IDO activity**
IDO activity was defined as kynurenine/tryptophan ratio and it was assessed in both plasma and urine at the end of the follow-up. The concentrations of tryptophan and kynurenines were measured using a high-throughput on-line solid-phase extraction-liquid chromatographic-tandem mass spectrometer (XLC-MS/MS)\textsuperscript{27}. Fifty microliters of lysate was pre-purified by automated on-line solid-phase extraction, using strong cation exchange (PRS, propylsulphonic) cartridges. Chromatographic separation of the analytes and deuterated analogues occurred by C18 reversed phase chromatography. Mass spectrometric detection was performed in the multiple reaction-monitoring mode using a quadrupole tandem mass spectrometer with positive electrospray ionization. Detection limit was 30 nmol/L for tryptophan and 1 nmol/L for kynurenines.

**Blood pressure measurement**
Prior to and every 2 weeks after transplantation, systolic blood pressure measurements were carried out in conscious animals with the tail-cuff plethysmography method (IITC Life Science, Woodland Hills, CA). Rats were trained to undergo blood pressure measurements 2 weeks before the first measurement. They were placed in restrainers while the temperature of the tail was maintained at 35-37°C. For each rat, the value was calculated as the mean of three to five consecutive measurements.
Markers of the renal damage

Prior to and every 2 weeks after transplantation, the urinary protein excretion was determined by nephelometry (Dade Behring III, The Netherlands) in 24 hour urine. In addition, plasma and urine creatinine levels were measured using i-STAT® System (Abbott, Den Hague, The Netherlands) and DCA Vantage Analyzer + kit (Siemens Healthcare Diagnostics Inc., Deerfield, IL, USA) and creatinine clearance was calculated using the formula creatinine clearance = (urine creatinine x urine flow) / (plasma creatinine x bodyweight).

To assess the degree of glomerular damage and transplant vasculopathy, paraffin sections were stained with periodic acid-Schiff (PAS). A qualified, independent pathologist semiquantitatively scored 50 glomeruli on a scale of 0 to 4 by light microscopy in a blinded fashion. Focal glomerular sclerosis (FGS) was scored as present when the collapse of capillary lumens, mesangial matrix expansion, hyalinosis and adhesion formation were present in the same quadrant. If 25% of the glomerulus was affected, it was scored as 1, 50% was scored as 2, 75% was scored as 3, and 100% as 4. The total FGS score was calculated by multiplying the score by the percentage of glomeruli with the same FGS score. The sum of these scores gives the total FGS score, ranging from 0 to 100%.

Transplant vasculopathy (TV) was assessed as intima/media ratio of intrarenal arteries with a diameter higher than 120 µm and evaluated blindly for the groups.

Immunohistochemistry and morphometry

Immunohistochemistry was performed on 3 µm paraffin sections. To evaluate renal damage after transplantation-infection procedure, sections were stained for α-smooth muscle actin (mouse monoclonal anti-α-smooth muscle actin, α-SMA, Sigma Chemical Co, St Louis, MO, USA) and macrophages (mouse monoclonal anti-ED-1, Serotec Ltd, Oxford, UK). Before the immunostaining procedure, paraffin sections were dewaxed and subjected to antigen retrieval by overnight incubation in 0.1M Tris/HCl buffer, pH 9.0, at 80°C. For immunohistochemistry, a two-step immunoperoxidase technique was used, according to standard techniques. Peroxidase activity was developed using 3, 3’-diaminobenzidine tetrachloride and H2O2. The expression of ED-1 and α-SMA by immunohistochemistry was measured using computer-assisted morphometry. A total of 40 (ED-1) or 30 (α-SMA) fields were evaluated per animal at a magnification of 200×. For ED-1, the number of positive cells per area was measured. For α-SMA, the total staining (excluding glomeruli and arteries) was divided by the area measured, and expressed as a percentage. An average score was calculated per section.
RNA isolation and real time PCR

Frozen kidney samples containing both the cortex and the medulla were homogenized, and RNA was isolated using a Qiagen kit (Qiagen, Venlo, The Netherlands), which included a DNAsel step. Integrity of RNA was determined using agarose gel electrophoresis, and the RNA concentration was measured spectrophotometrically at 260 nm. RNA (1 μg) was reverse-transcribed, and cDNA was further used to analyze rat IDO, foxp3, hIDO, TGF-β, renin and ACE gene expression using a real-time PCR protocol, as described previously28. Sequence-specific PCR primers were purchased from Biolegio (Nijmegen, The Netherlands). The sequences of the primers used were as follows: rat IDO forward: 5'-CTCCGAGAAGAAGTCGAGAA-3', reverse 5'-TTCTCCAGACTGGCAGCTAT-3'; human IDO forward: 5'-TCATGGAGATGTCCGTAAGG-3', reverse: 5'-GCCAAGACACAGTCTGCATA-3'; foxp3 forward: 5'-CCACACCTCCTCTTTCCCTTT-3', reverse: 5'-TGACTAGGGCAGCTGTAGGC-3'; TGF-β forward: 5'-ATACGCCTGAGTGGCTGTCT-3', reverse: 5'-TGGGACTGATCCATTGATT-3'; renin forward: 5'-CTGTGCATACTGGCTCTCCA-3', 5'-reverse: GGCTTGGCCTAAAACCTAGGG-3' and ACE forward: 5'-GTGTTGTGGAACGATACGC-3', reverse: 5'-CCTTCTTTATGATCCCGCTTG-3'. All values were expressed as saline = 100% and standardized on GAPDH.

Statistical Analyses

Data are presented as mean ± SEM. Area under the curve (AUC) for blood pressure and proteinuria were calculated and expressed as arbitrary units. Significance was tested with one way ANOVA followed by a least significant difference post hoc test. The relationships between ED-1 and α-SMA/FGS and between rIDO and foxp3/TGF-β were calculated using Spearman’s nonparametric correlation. Difference was considered significant at p<0.05.
Results

Clinical parameters

During the follow-up, one rat had to be killed prematurely (in week 8 after transplantation) in the RGD-AdTL treated group due to a deterioration of its general condition, as indicated by piloerected fur and severe weight loss (6% weight loss within 2 days). All rats in the RGD-AdTIDO and saline group survived until the end of the experiment. The prematurely killed rat was excluded from all histological and biochemical analyses described below.

For the long-term follow-up (12 weeks), the following groups were studied: saline group (n = 5), RGD-AdTL group (n = 5) and RGD-AdTIDO group (n = 7).

The treatment with RGD-AdTIDO significantly increased body weight of recipients in comparison with rats treated with RGD-AdTL or saline, starting from week 6 after transplantation (Figure 1A).

Following transplantation, systolic blood pressure increased gradually from a mean of 129.0±2.5 mmHg and 128.8±1.8 mmHg to 162.6±7.7 mmHg and 154.1±10.3 mmHg in the allograft recipients treated with saline and RGD-AdTL, respectively. In contrast, the RGD-AdTIDO treated group only showed a modest increase in systolic blood pressure from 128.6±1.4 mmHg to 136.5±1.8 mmHg. RGD-AdTIDO treatment significantly reduced the increase in systolic blood pressure compared to the RGD-AdTL and saline (p<0.05; Figure 1B).
Urinary protein excretion, plasma creatinine, creatinine clearance

Proteinuria, a hallmark of progressive renal injury, increased in the saline and RGD-AdTL treated groups from respectively week 6 and 8 after transplantation and continued to increase thereafter. RGD-AdTIDO treatment attenuated the increase of urinary protein excretion in time and significantly attenuated the increase observed in the saline group (p<0.05) (Figure 1C).

The values of plasma and urine creatinine and the creatinine clearance are summarized in Table 1. RGD-AdTIDO group showed a trend toward decreased serum creatinine levels, increased levels of urinary creatinine and increased creatinine clearance, compared to the RGD-AdTL and saline treatment, however without reaching statistical significance.
Table 1. Serum and urinary creatinine, creatinine clearance, serum and urinary kynurenine and tryptophan, serum and urinary ratio of kynurenine to tryptophan

<table>
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<tr>
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<th>Saline</th>
<th>RGD-AdTL</th>
<th>RGD-AdTIDO</th>
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<tr>
<td>Serum creatinine (µmol/l)</td>
<td>45.00 ± 5.26</td>
<td>49.00 ± 7.38</td>
<td>40.00 ± 4.26</td>
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<td>Urine creatinine (µmol/l)</td>
<td>6.12 ± 1.07</td>
<td>4.40 ± 0.40</td>
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<td>Cr. clearance (ml/min/kg)</td>
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<td>Serum kynurenine (µmol/l)</td>
<td>1.70 ± 0.76</td>
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<td>Serum tryptophan (mmol/l)</td>
<td>0.07 ± 0.01</td>
<td>0.08 ± 0.04</td>
<td>0.07 ± 0.00</td>
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<td>Ratio kyn/trp</td>
<td>24.90 ± 2.58</td>
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<td>Urinary kynurenine (µmol/l)</td>
<td>1.94 ± 0.39</td>
<td>1.11 ± 0.32</td>
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<td>Urinary tryptophan (mmol/l)</td>
<td>0.009 ± 0.005</td>
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<tr>
<td>Ratio kyn/trp</td>
<td>358.04 ± 92.85</td>
<td>320.70 ± 111.87</td>
<td>449.20 ± 47.32</td>
</tr>
</tbody>
</table>

kyn, kynurenine; trp, tryptophan; cr, creatinine

**Effects of renal IDO expression on plasma and urinary kyn/trp levels**
Neither the serum levels of kynurenines and tryptophan, nor those of kyn/trp ratio were significantly different between groups (Table 1). Urinary kyn/trp ratio was slightly higher in the IDO group, however without reaching statistical significance (Table 1).

**Development of transplant vasculopathy, focal glomerulosclerosis, interstitial fibrosis, and interstitial macrophage influx**
Chronic transplant dysfunction (CTD) in the rat is characterized by transplant vasculopathy, mild glomerular basement membrane thickening and sclerosis, interstitial fibrosis and severe tubular atrophy.
Transplant vasculopathy (TV) is the main feature of CTD and contributes to the graft loss. The incidence of TV, evaluated as intima/media ratio, was significantly decreased by IDO treatment as compared to RGD-AdTL and saline group (2.7 ± 0.2 vs. 3.8 ± 0.4 and 3.6 ± 0.4, respectively, p<0.05; Figure 2).
Figure 2. Transplant vasculopathy three months after transplantation. Treatment with RGD-AdTIDO significantly ameliorated transplant vasculopathy in comparison with RGD-AdTL and saline (A). *p<0.05 vs RGD-AdTL; # p<0.05 vs saline; representative pictures of RGD-AdTL (B), saline (C), RGD-AdTIDO (D); transplant vasculopathy was expressed as intima/media ratio.
Furthermore, CTD was associated with an abundant influx of macrophages in the interstitium in both RGD-AdTL and saline groups as quantified by ED-1 positive cells. Treatment with RGD-AdTIDO markedly reduced the influx of macrophages into the graft (100% and 89% compared to 52%, respectively, p<0.05; Figure 3).

PAS staining revealed mild FGS and interstitial α-SMA expression in the allografts of RGD-AdTL and saline recipients. RGD-AdTIDO treatment showed a trend towards decreased FGS and α-SMA score, although this did not reach statistical significance (Figures 4, 5). As expected, a positive correlation was found between the expression of α-SMA and ED-1 positive cells and between FGS and ED-1 positive cells across all three groups (R=0.587, p<0.05 and R²=0.558, p<0.05).

**Figure 3. The influx of macrophages into the interstitium.** RGD-AdTIDO treatment significantly decreased the influx of macrophages into the graft. *p<0.05 vs RGD-AdTL; # p<0.05 vs saline; ED-1, marker for macrophages
Figure 4. Focal glomerulosclerosis three months after transplantation. Treatment with RGD-AdTIDO did not influence significantly the focal glomerulosclerosis (A). Representative pictures of RGD-AdTL (B), saline (C), RGD-AdTIDO (D)
To establish whether IDO treatment affected the number of regulatory T-cells in the kidney, the expression levels of its marker Forkhead box p3 (foxp3) were evaluated by real time PCR. RGD-AdTIDO treatment up-regulated the level of foxp3 mRNA significantly in comparison with the saline group (177.2 ± 6.3% for RGD-AdTIDO vs 100 ± 13.5% for saline, p<0.05; Figure 6A).

Additionally, expression of rat IDO mRNA (rIDO) was quantified to assess whether transfection with hIDO influenced the expression of recipient IDO. RGD-AdTIDO treatment up-regulated rIDO compared to the saline group (110.8 ± 1.5% vs 100 ± 0.5%, p<0.05; Figure 6B).

TGF-β plays an important role in the generation of foxp3+ T regulatory cells and induction of tolerance. We observed a significant increase in the mRNA expression of TGF-β in IDO treated kidney, compared to both saline and RGD-AdTL group (105.5 ± 1.8% vs 100 ± 1.8% and 97.4 ± 3.2%, respectively, p<0.05; Figure 6C). Moreover, there...
was a positive correlation between rIDO and foxp3 mRNA (R=0.556, p<0.05) and between rIDO and TGF-β mRNA (R=0.653, p<0.05).

Expression of renal RAAS components mRNA

As IDO gene therapy markedly attenuated the increase in systolic blood pressure following transplantation, without indications for increased systemic IDO activity. In search of a mechanism for this effect, we evaluated the mRNA expression of renin, angiotensin II receptor type 1 (AT1R) and angiotensin converting enzyme (ACE) in the transplanted graft. Real time PCR analysis showed no differences in mRNA expression of

Figure 6. The effect of RGD-AdTIDO treatment on the mRNA levels of foxp3, ratIDO and TGF-β. RGD-AdTIDO treatment significantly increased the level of foxp3 (A), rat IDO (B) and TGF-β (C) mRNA. *p<0.05 vs RGD-AdTL; # p<0.05 vs saline; values were standardized on GAPDH, saline was expressed as 100%.
renin (Figure 7A) and AT1R (data not shown) between the experimental groups, whereas mRNA of ACE was slightly down-regulated in the RGD-AdTIDO group in comparison with saline and RGD-AdTL groups (95.9 ± 1.2% vs 100 ± 1.7% and 101.5 ± 0.8%, respectively, p<0.05; Figure 7B).

Figure 7. The effect of RGD-AdTIDO treatment on the mRNA levels of renin and ACE. RGD-AdTIDO did not affect the levels of renin mRNA (A), however it significantly down-regulated the level of ACE mRNA (B). *p<0.05 vs RGD-AdTL; # p<0.05 vs saline; values were standardized on GAPDH, saline was expressed as 100%. ACE, angiotensine converting enzyme.
Discussion

The major finding of the present study is that adenovirus-mediated gene therapy with IDO attenuates the key features of CTD, as documented by decreased proteinuria, decreased systolic blood pressure, and reduced incidence of transplant vasculopathy. This is accompanied by a reduced infiltration of macrophages into the transplanted graft, and an increased foxp3 mRNA and TGF-β mRNA expression in the IDO treated animals.

Several mechanisms may be involved in the beneficial effects of IDO on the development of CTD found in our study. A first mechanism may involve an increased expression of TGF-β and foxp3 mRNA, the later one being a marker of active regulatory T cells (Treg)\(^3\)). Previously, an immunoregulatory mechanism involving TGF-β and Treg have been identified in mice recipients that had spontaneously accepted renal allografts\(^3\)). In those mice, early escape from cellular rejection was associated with regulation of graft-reactive T cells by a transient TGF-β-mediated mechanism. TGF-β is a known stimulator of maturation of CD4+CD25+ Treg. Accumulating data support the role of Treg in long-term allograft acceptance\(^3\);\(^3\);\(^4\);\(^5\);\(^6\), while TGF-β appears to be an essential intermediary in this process\(^3\);\(^2\);\(^3\);\(^7\). In the same model of spontaneous renal graft acceptance, long-term escape from rejection was associated with regulation of graft-reactive T cells by IDO, while TGF-β-mediated regulation being lost in time. The source of IDO in this model was the regulatory dendritic cells (DCs) which expressed IDO upon the stimulation of Treg. Conversely, it has also been shown that IDO+ DCs are capable of stimulating the development of Treg\(^3\);\(^8\);\(^9\). The most probable scenario in our study is that the initial expression of IDO in the renal graft led to the formation of Treg, with TGF-β taking over the stimulation of Treg formation later in time. Another possibility is that the increased rat IDO is the direct or additional factor enhancing the foxp3 and TGF-β mRNA expression in the chronic phase after transplantation. In support to this hypothesis come the positive correlations we found, on one hand between the rat IDO and foxp3, and on the other hand between rat IDO and TGF-β mRNA levels.

The most important risk factor for development of CTD is the incidence of acute rejection\(^1\);\(^0\). Previously, we demonstrated beneficial effects of IDO gene therapy in protection against acute rejection of the renal graft\(^2\);\(^1\). Therefore, another possible mechanism of the beneficial effects of IDO in CTD is the influence of IDO on acute, immune-mediated graft injury, on top of the effect of the low-dose Cyclosporine.

IDO may have also influenced the outcome of the kidney transplantation in our study by interfering with the regulation of blood pressure and proteinuria. CTD is characterized by a relative slow but variable rate of decline in renal function. This is often
found in combination with proteinuria and aggravation of pre-existing or de novo hypertension\(^{40,41}\). High blood pressure, along with proteinuria, has been associated with reduced graft survival\(^10\). Hypertension may promote atherosclerosis within renal blood vessels or glomerular hypertension, which can increase glomerular permeability and consequently enhance protein trafficking and thus proteinuria\(^10\). In our study, we observed that local gene therapy with IDO significantly decreased systolic blood pressure and proteinuria as compared to the saline group. Hofmann et al. showed that metabolisation of tryptophan to kynurenines by the IDO expressed in endothelial cells contributes to arterial vessel relaxation and thereby the control of blood pressure, during the inflammation and sepsis. This was mediated by activation of the adenylate and soluble guanylate cyclase pathways\(^{42,43}\). Moreover, kynurenine administration decreases blood pressure in a dose-dependent manner in spontaneously hypertensive rats and it has been proposed as a novel endothelium derived vascular relaxing factor\(^42\). There is no indication however, that this mechanism is also involved in the hypertension associated with CTD. In our study, the systemic levels of tryptophan and kynurenines were not different in the rats receiving local IDO gene therapy as compared to the controls. Nevertheless, it is well known that blockade of the renin-angiotensin system (RAS) with angiotensin-converting enzyme (ACE) inhibitors and angiotensin II (Ang II) receptor blockers slow down the progression of chronic allograft dysfunction\(^44\). ACE inhibitors are often used to achieve adequate control of hypertension associated with CTD. Another beneficial effect of ACE inhibitors is a significant reduction of proteinuria\(^45\). Both mentioned effects of ACE inhibitors can affect the long-term graft survival. Using real time PCR we found that IDO did not influence the mRNA expression of renin and type 1 ATII receptor in the graft, however, interestingly, significantly down-regulated ACE mRNA in RGD-ADTIDO group compared to both untreated groups. Thus, rather than a direct effect of kynurenines on vasodilatation, reduction of blood pressure in IDO treated animals may depend on ACE expression.

Although IDO treatment did not significantly affect the incidence of FGS and expression of \(\alpha\)-SMA in our model, it did ameliorate the development of transplant vasculopathy (TV) and the influx of macrophages into the graft. TV is a specific lesion of chronic allograft nephropathy, which leads to impaired perfusion and subsequent allograft dysfunction\(^2\). Endothelial dysfunction and damage plays an important role in the initiation of TV, because endothelial cells respond to inflammatory cytokines produced by activated T cells and/or macrophages and because endothelial cells are the first foreign cells that come in contact with the alloreactive T cells of the recipient\(^{46,47}\). Therefore, the
improvement of TV in our model might be a consequence of reduced macrophages influx into the graft, initiated by IDO.

Crucial for long-term immunomodulation via gene therapy is the use of a vector able to induce high-level transgene expression and to drive expression of the therapeutic gene for a prolonged period of time. Our modified adenovirus that has an Arg-Gly-Asp (RGD) motif inserted in the HI loop of the fiber knob represents a successful strategy to transduce the renal graft, as we previously documented\textsuperscript{23}. However, transgene expression lasted in the absence of any immunosuppression for only 2-3 weeks\textsuperscript{23}. Interestingly, when IDO was inserted into the adenovirus and delivered to the kidney using the same technique, a clear increase in the GFP expression in the RGD-AdTIDO compared to RGD-AdTL group at day 7, was found\textsuperscript{21}. This observation suggests that IDO-mediated inhibition of T-cell infiltration or function prolongs the duration of human IDO expression in the kidney by protection of the infected cells against immune clearance. Thus, most probably, transgene IDO expression lasts longer than 3 weeks in the present experimental setting. However, no human IDO was found at the end of the experiment.

In summary, we show here for the first time that local adenoviral gene therapy with IDO has beneficial effects on the key features of CTD. This may be due in part to a TGF-β mediated and in part to a direct stimulation of Treg formation, in addition to interference with the regulation of blood pressure and proteinuria. Thus, gene therapy with IDO constitute a new appealing strategy in the armamentarium against CTD.
Reference List


