Gene therapy with adenovirus-delivered indoleamine 2,3-dioxygenase improves renal function and morphology following allogeneic kidney transplantation in rat


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Abstract

Background: Indoleamine 2.3-dioxygenase (IDO), the rate limiting enzyme in the tryptophan catabolism, has recently emerged as an important immunosuppressive enzyme involved in the regulation of both physiologic (maternal tolerance), as well as pathologic (neoplasia, autoimmune diseases, asthma) processes. Accumulating evidence points to a role for IDO in suppressing T-cell responses, thereby promoting tolerance. Here we investigate the effects of IDO on the acute rejection of the transplanted kidneys, using an adenovirus-mediated gene delivery approach.

Methods: The experiments were performed in a rat Fisher to Lewis acute renal rejection model. RGD modified adenovirus carrying IDO gene (RGD-AdTIDO, n=9) or RGD modified adenovirus carrying gene for GFP (RGD-AdTL, n= 8) were injected into the renal artery of the donor kidney before transplantation. A group receiving saline (n= 8) served as control. Rats were sacrificed after 7 days.

Results: Successful gene delivery was confirmed with real time polymerase chain reaction and immunohistochemistry. RGD-AdTIDO significantly decreased elevated plasma creatinine (93.7 ± 18.9 µmol/l) compared to the RGD-AdTL (248.2 ± 43.6 µmol/l) and saline (228.3 ± 46.4 µmol/l) treated rats. Moreover, RGD-AdTIDO therapy diminished the infiltration of cytotoxic CD8+ T cells and macrophages into the graft and reduced renal interstitial pre-fibrosis. Also, it limited the up-regulation of kidney injury molecule-1, interleukin (IL)-2, IL-17 and transforming growth factor-β mRNA expression, and increased foxp3 mRNA expression compared to controls.

Conclusion: RGD-AdTIDO therapy improves renal function and morphology in a clinically relevant model of acute rejection.
Introduction

Although kidney transplantation is currently the treatment of choice for most patients with end-stage renal failure, its full success is hampered by the rejection of the grafted organ. Currently, systemic immunosuppressive therapy is employed to reduce the risk of graft rejection, at the cost of serious side-effects, such as the development of infections, malignancies and cardiovascular morbidity. Therefore, strategies aiming at the minimization/avoidance of systemic immunosuppression, such as local immunosuppression in the renal graft or induction of transplant-specific tolerance, are currently being explored as alternatives to systemic immunosuppression.

Amongst the candidates for achieving this goal, molecules governing foetal-maternal tolerance, such as indoleamine 2,3-dioxygenase (IDO), may provide useful strategies of intervention. IDO is the rate-limiting enzyme in the catabolism of the essential amino acid tryptophan into L-kynurenine and its downstream metabolites, kynurenic acid, 3-hydroxykynurenine, 3-hydroxyantracilic acid and quinolinic acid. For many years, IDO has been known as an enzyme involved in host defense against infections. With their seminal study, Munn and Mellor showed that IDO expression in the placenta is crucially involved in the foetal-maternal tolerance because the inhibition of IDO by 1-methyl tryptophan induced the rejection of the foetus.

The mechanisms behind the immunosuppressive or tolerogenic effects of IDO are complex and still incompletely understood. Presently, three mechanisms of action of IDO are considered: tryptophan depletion, the generation of immunosuppressive metabolites and the induction of regulatory T cells. IDO is constitutively expressed in organs with large areas of mucosal tissue (lung, gut, placenta), as well as in antigen-presenting cells, supporting a role in the naturally occurring immune surveillance. IDO steers the phenotype of antigen-presenting cells towards immunosuppression, as reported in dendritic cells. Moreover, inducible IDO expression (mostly by interferon-g stimulation) has been described in fibroblasts, macrophages, endothelial cells and tumor cells, with the latter using IDO expression as a mechanism to avoid immune recognition and destruction. In addition, local IDO expression has been reported as a protective mechanism in several diseases with an immune component, such as asthma, allergic airway inflammation and diabetes.

These properties have made IDO an appealing target for intervention in animal models of organ transplantation. Nevertheless, the literature on IDO and kidney transplantation is limited. In a mouse model of spontaneous kidney allograft acceptance, Orosz et al. reported evidence for an IDO-regulatory dendritic cell mechanism of late
acceptance, whereas early acceptance was mediated by transforming growth factor (TGF)-β and regulatory T cells. In human kidneys with acute rejection, the expression of IDO has been reported\textsuperscript{23}; however, the role of IDO in this context is unclear. In addition, the therapeutic potential of IDO in kidney transplantation has not yet been explored. Previously, we developed an adenovirus-based gene delivery technique that enables us to specifically target genes to the transplanted kidney\textsuperscript{24}. In the present study, we utilize this technique to explore the capacity of IDO to inhibit acute rejection following kidney transplantation in rats.
Material and Methods

Experimental design

Experiments were performed in a model of acute kidney allograft rejection, in which inbred male Fisher (F344 NHsd) rats were used as donors and male Lewis (SsnHsd) rats were used as recipients. Body weight 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 described24. Briefly, donor kidneys were infused with adenovirus solution via the renal artery [4x10^{11} viral particles (VP) per animal] 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. At the end of the transplantation procedure, a contralateral nephrectomy was performed, so that the renal function exclusively relied on the graft. Also, a blood sample from the tail vein of the recipient was taken before starting the transplantation. Animals did not receive any additional immunosuppressive treatment.

Three groups of animals were included. In a first group donor kidneys were infused with an RGD-adenovirus carrying the reporter gene for green fluorescent protein (GFP) (T, tracking) and the gene for human IDO (RGD-AdTIDO group, n=9). In a second group an adenovirus carrying the reporter genes GFP and luciferase was used (RGD-AdTL group, n=8). An additional group receiving saline was included as control (saline group, n=8). Animals were sacrificed at day 7. Because, as reported in the literature, there is no significant raise in plasma creatinine levels approximately 1 week after syngeneic transplantation26, we did not include additional isotransplantation groups in the present study.

At termination, 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 immunohistochemistry. A second slice was snap-frozen in liquid nitrogen, stored at 80ºC and further used for mRNA isolation.

Adenoviral vectors

RGD-modified adenoviruses27 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. Using homologous recombination in Escherichia coli BJ5183, the shuttle was integrated into the RGD adenovirus plasmid pVK503, 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 293 cells and purified by double CsCl density centrifugation. The amount of viral particles VPs 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.

**Western blotting**

IDO protein expression by the RGD-AdTIDO vector was confirmed in transduced 293 cells by Western blot analysis, as previously described. Briefly, transduced cells were washed 3 times using phosphate-buffered saline (PBS) and subsequently lysed in 800 µl of M-PER Mammalian protein extraction reagent (Pierce Biotechnology, USA). Protein concentrations were determined using Bio-Rad protein assay. Equal amount of protein in SDS-PAGE sample buffer was separated on 4-20% PAA-SDS gels. After transfer to nitrocellulose membranes (Amersham, UK), protein samples were incubated with primary antibody against IDO (Millipore, The Netherlands) and thereafter with a horseradish peroxidase-conjugated rabbit anti-mouse antibody (Dako, USA). Signals were detected by chemiluminiscent detection using ECL (Amersham, The Netherlands).

**High-performance liquid chromatography (HPLC) assay for functional hIDO expression**

To verify the functionality of the hIDO construct, IDO activity was measured *in vitro* by quantifying metabolization of tryptophan to kynurenines in 293 cells transduced with RGD-AdTIDO [multiplicity of infection (MOI) of 50], RGD-AdTL (MOI of 50) and in untransduced cells. Cells were cultured for 24 hours and the concentrations of tryptophan and kynurenines were measured in the cell lysates using a high-throughput on-line solid-phase extraction-liquid chromatographic-tandem mass spectrometer. 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. The same method was employed for the measurement of tryptophan and kynurenine levels in serum from day 7.

**Plasma creatinine**

Plasma creatinine levels were determined as a measure of renal function, in blood samples collected on day 0 and at the end of the study (i.e. day 7), using the enzymatic colorimetric assay CREA plus (Roche Diagnostics, Mannheim, Germany).

**Immunohistochemistry and morphometry**

Immunohistochemistry was performed on 3 μm paraffin sections. To confirm successful gene delivery into the donor kidney, a GFP immunostaining with rabbit polyclonal anti-GFP antibody (Molecular Probes, Leiden, the Netherlands) was performed. To evaluate the 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), macrophages (mouse monoclonal anti-ED-1, Serotec Ltd, Oxford, UK) and cytotoxic T lymphocytes (mouse monoclonal anti-CD8, a generous gift from Dr. J.L Hillebrand, UMCG, the Netherlands). Before the immunostaining procedure, paraffin sections were dewaxed and subjected to antigen retrieval by microwave induced heat in 0.1M Tris/HCl, pH 9.0 (GFP) or in 1mM EDTA buffer, pH 8.0 (CD8), and overnight incubation in 0.1M Tris/HCl buffer, pH 9.0, at 80°C (α-SMA, ED-1). 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 GFP, ED-1 and α-SMA by immunohistochemistry was measured using computer-assisted morphometry. Total of 30 (GFP, α-SMA) or 40 (ED-1) fields were evaluated at a magnification of 200×. For GFP, three sections per rat were stained. The GFP staining was divided by the area measured and expressed as a percentage. 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. CD8+ cells were counted in 30 fields using an ocular grid at 400×magnification and
expressed as the number of positive cells per field. Evaluation of the stainings and morphometric analysis were performed in a blinded manner.

**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 DNAse step. Integrity of RNA was determined using agarose gel electrophoresis, and the RNA concentration was measured spectrophotometrically at 260 nm. RNA (1 μg, except for foxp3, where 3 μg was used) was reverse-transcribed, and cDNA was further used to analyze rat kidney injury molecule-1 (KIM-1), transforming growth factor (TGF)-β, interleukin (IL)-2, foxp3 and IL-17 and hIDO gene expression using a real-time PCR protocol, as described previously (32). Sequence-specific PCR primers were purchased from Biolegio (Nijmegen, The Netherlands). The sequences of the primers used were as follows: KIM-1 forward: 5’-GTCTGTATTGTTGCGGAGTG-3’, reverse: 5’-GGTTCTTGTGTGGAGGACTTG-3’ (106 bp); TGF-β forward: 5’-ATACGCCTGAGTGCCGTCTG-3’, reverse: 5’-TGGGACTGATCCCATTGATT-3’ (153 bp) and IL-2 forward: 5’-ATGCAGCTCGCATCCTGTT-3’, reverse: 5’-CAATTCTTGCGCTTGGCCTTG-3’ (740 bp); foxp3 forward: 5’-GCACAAGTGGCTTTGCGAGTG-3’, reverse: 5’-TGTCGTGGTTGCGAGACAG-3’ (572 bp); IL-17 forward: 5’-ATGTGCGTGGTGGTGCTGCTGTA-3’, reverse: 5’-TTAGGACGCGTCGACCCAGTAAG-3’ (453 bp); human IDO forward: 5’-TCATGGAGATGTCCCGTAAAGG-3’, reverse: 5’-GCCAAGACACAGTCTGCATA-3’ (111 bp).

**Statistical Analyses**

Data are presented as the mean ± SEM. Significance was tested with one way ANOVA followed by a least significant difference post hoc test. p<0.05 was considered statistically significant.
Results

Expression of a functional hIDO with the RGD-AdTIDO construct

First, expression of IDO protein by the RGD-AdTIDO construct was verified \textit{in vitro} in 293 cells. An expected 42 kDa protein band was detected in the cells infected with RGD-AdTIDO, while cells transduced with the RGD-AdTL construct or untransduced control cells did not show any IDO expression (Figure 1). Further, functionality of the IDO construct was verified by assessing \textit{in vitro} the metabolization of tryptophan to kynurenines in 293 cells transfected with RGD-AdTIDO, RGD-AdTL and in untransduced cells. As shown in Table 1, IDO expression by the RGD-AdTIDO both depleted tryptophan content and increased kynurenine concentration in cell lysates as compared to the controls, confirming IDO functionality.

Figure 1. IDO protein expression detected by western blotting in 293 cells. 293 cells were cultured either in the absence (control) or presence of RGD-AdTL or RGD-AdTIDO, respectively. A 42 kDa band corresponding to the size of IDO protein was detected in lysates of cells transduced with RGD-AdTIDO, whereas no band was detected in cells transduced with RGD-AdTL and in untransduced control cells.

Transgene expression in the donor kidneys and systemic kynurenine/tryptophan ratio

To confirm gene delivery with the RGD-adenovirus we first investigated the expression of the hIDO gene in the transplanted kidneys, at 7 days after transplantation. The hIDO mRNA was only found in the kidneys of rats transduced with RGD-AdTIDO, whereas no transcript was observed in RGD-AdTL treated or in saline treated kidneys (Figure 2A). Furthermore, to identify the transduced cells, we performed an immunostaining for GFP in all three groups of animals (Figure 2B). There was substantial GFP expression in the RGD-AdTIDO transduced kidneys (2.3 ± 0.2%) and GFP expression to a lesser extent in the RGD-AdTL group (0.5 ± 0.1%) (p<0.01), whereas GFP expression was absent in the saline-treated animals. Expression was found mostly in the interstitium, in cells resembling...
fibroblasts, as previously reported\textsuperscript{24}. We also investigated whether our therapy affected the systemic kynurenine levels. No significant difference was found in the kynurenine/tryptophan ratio between the saline (66.9 ± 6.5μmol/mmol), RGD-AdTL (49.6 ± 9.5μmol/mmol) and RGD-AdTIDO (58.2 ± 15.4μmol/mmol) groups (p<0.05).

<table>
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<tr>
<th>Tryptophan (μmol/l)</th>
<th>Kynurenines (μmol/l)</th>
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<tr>
<td>control</td>
<td>0.48 ± 0.08</td>
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<tr>
<td>RGD-AdTL</td>
<td>0.65 ± 0.17</td>
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<tr>
<td>RGD-AdTIDO</td>
<td>0.12 ± 0.05*</td>
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The concentration of tryptophan and kynurenines was determined using HPLC in lysates of 293 cells transduced with RGD-AdTIDO (MOI of 50), RGD-AdTL (MOI of 50) and in untransduced control. <LOD, below the limit of detection (0.001 μmol/l), *p<0.05 vs. RGD-AdTL and control. #p<0.01 because the value is more than three SDs above detection limit.

\textit{RGD-AdTIDO therapy attenuates renal transplant dysfunction}

Renal function was assessed by measurement the plasma creatinine in blood samples collected before transplantation (day 0) and at the end of the study (day 7) (Figure 3). Creatinine level in plasma of recipients rats before surgery amounted 34.8 ± 6.7 μmol/l. Renal transplantation induced a substantial increase in plasma creatinine in the saline treated group (228.3 ± 46.4 μmol/l), which was not statistically different from the level found in the RGD-AdTL group (248.2 ± 43.6 μmol/l). The RGD-AdTIDO therapy significantly attenuated the increase in plasma creatinine compared to both untreated and RGD-AdTL groups (93.7 ± 18.9 μmol/l, p<0.05).
Figure 2. Quantification and localization of transgene expression 7 days after viral transduction of the donor kidney. (A) Real-time PCR was performed using primers specific for hIDO. mRNA was isolated from frozen kidneys, followed by hIDO gene amplification. Data document expression of hIDO mRNA solely in the kidneys of rats transduced with RGD-AdTIDO. (B) Immunohistochemistry for the GFP transgene was performed on paraffin sections using a rabbit polyclonal anti-GFP antibody followed by a two-step immunoperoxidase assay. Three sections per rat were evaluated using computer-assisted morphometry. Significantly more GFP staining was found in the RGD-AdTIDO group compared to the RGD-AdTL group (*p<0.01), whereas saline-treated rats were devoid of GFP expression. (C) Representative images from GFP immunostaining. (a) Saline-treated kidneys lack GFP expression. (b) Limited GFP staining is observed in RGD-AdTL transduced kidneys (brown staining, arrows). (c) Substantial GFP expression (brown staining, arrows) is observed in the RGD-AdTIDO transduced kidneys. Scale bars=100µm.

**RGD-AdTIDO therapy reduces graft CD8+ T lymphocyte and macrophage infiltration**

The effects of IDO gene therapy on graft infiltration with CD8+ T cells and macrophages in the renal interstitium were examined using immunohistochemistry for CD8+ and ED-1, respectively. In saline and RGD-AdTL groups, a substantial accumulation of CD8+ T cells (Figure 4A) and macrophages (Figure 4B) was observed. RGD-AdTIDO therapy significantly diminished the interstitial infiltration of CD8+ T cells and macrophages.
Figure 3. **IDO effect on increased plasma creatinine.** Creatinine plasma levels were measured using the enzymatic colorimetric assay CREA plus in blood samples collected before transplantation and at the end of the experiment (day 7). IDO therapy significantly decreased the levels of plasma creatinine compared to saline and RGD-AdTL groups (*p<0.05 versus saline and RGDAdTL; #p<0.05 versus RGD-AdTIDO; tx, transplantation).

![Creatinine Levels](image)

Figure 4. **Inhibitory effect of IDO on interstitial inflammatory cell infiltration after allotransplantation.** Infiltration with CD8+ T cells and macrophages was studied using immunohistochemistry with a mouse monoclonal anti-CD8 and mouse monoclonal anti-ED-1 antibody, respectively. A two-step immunoperoxidase assay was used. CD8+ cells were counted manually in 30 fields at 400 magnification. The number of ED-1+ cells was estimated in 40 fields at 200 magnification, using computer-assisted morphometry. Local IDO gene therapy significantly reduced the amount of CD8 positive cells (A) and ED-1 positive cells (B) compared to saline and RGD-AdTL groups. *p<0.05 versus saline and RGD-AdTL.
RGD-AdTIDO therapy reduces the expression of damage parameters α-SMA and KIM-1

Because proliferation of myofibroblasts expressing the α-smooth muscle actin (α-SMA) is widely recognized as a key event in early transplantation-related injury,[33] its expression was studied as a marker of renal pre-fibrosis. With immunohistochemistry, we observed considerable interstitial α-SMA staining in the saline and RGD-AdTL groups (15.4 ± 0.4% and 13.6 ± 0.9%, respectively). RGD-AdTIDO therapy significantly decreased the expression of α-SMA (7.1 ± 0.9%) compared to both control groups (Figure 5A).

Increased transcription of kidney injury molecule-1 (KIM-1) is a specific biomarker for early tubular injury after kidney transplantation.[34] Therefore, we measured expression of KIM-1 mRNA by real-time PCR. RGD-AdTIDO therapy significantly down-regulated the elevated expression of KIM-1 mRNA [0.4 ± 0.1 arbitrary units (au)] as found in saline and RGD-AdTL groups (2.0 ± 0.4 and 2.0 ± 0.3 au, respectively) (Figure 5B).

**Figure 5. Treatment with IDO reduces renal damage.** (A) Interstitial pre-fibrosis was assessed by immunohistochemistry for α-SMA using a mouse monoclonal antibody, followed by a two-step immunoperoxidase assay. The stained area was measured by computer-assisted morphometry, excluding the glomerular area and the blood vessels from the measurement. IDO gene therapy significantly diminished the expression of α-SMA compared to saline and RGD-AdTL groups. (B) Tubular epithelial cell injury was assessed by measuring the expression of KIM-1 mRNA using real-time PCR. IDO gene therapy significantly down-regulated the expression of KIM-1 mRNA. *p<0.05 versus saline and RGD-AdTL.
RGD-AdTIDO therapy down-regulates IL-2, IL-17 and TGF-β mRNA and up-regulated foxp3 mRNA

Cytokines are key mediators in the induction and effector phases of the immune and inflammatory responses in kidney transplantation. The effect of RGD-AdTIDO therapy on the mRNA levels of the cytokines IL-2, IL-17 and TGF-β was measured using real-time PCR. RGDAdTIDO therapy markedly down-regulated the increased expression of IL-2 mRNA (0.36 ± 0.1 au) compared to the saline (0.97 ± 0.05 au) and RGD-AdTL (1.19 ± 0.12 au) groups (Figure 6A). RGD-AdTIDO also significantly diminished the levels of IL-17 mRNA (saline: 1.32 ± 0.09 au; RGD-AdTL: 1.48 ± 0.09 au; RGD-AdTIDO: 0.65 ± 0.1 au) (Figure 6B) and limited the up-regulation of TGF-β mRNA (saline: 3.50 ± 0.16 au, RGD-AdTL: 3.73 ± 0.24 au, RGD-AdTIDO: 2.68 ± 0.19 au; notably, the levels of TGF-β found in normal Fisher kidneys were 1.41 ± 0.1 au (Figure 6C). By contrast to the effects on the above mentioned cytokines, significantly higher levels of the regulatory T-cell marker foxp3 mRNA were found the RGD-AdTIDO group (1.70 ± 0.18 au) compared to the saline (0.47 ± 0.06 au) and RGD-AdTL (0.49 ± 0.08 au) groups (Figure 6D).
Figure 6. Effect of IDO on the mRNA levels of IL-2, IL-17, TGF-β and foxp3. mRNA levels of IL-2, IL-17, TGF-β and foxp3 were measured using real-time PCR. Local gene therapy with IDO inhibited the up-regulation of IL-2 (A), IL-17 (B) and TGF-β (D) mRNA compared to saline and RGD-AdTL groups, and upregulated foxp3 mRNA levels (C). *p<0.05 versus saline and RGD-AdTL.
Discussion

The major finding of the present study is that adenovirus-mediated gene therapy with IDO attenuates the early functional and morphological deterioration of the kidney associated with allotransplantation in rats. This is demonstrated by significantly decreased plasma creatinine levels in the RGD-AdTIDO-treated animals, which were accompanied by a substantial reduction in the tubular injury and interstitial pre-fibrosis, as well as a marked reduction of renal inflammation.

The pathogenesis of the acute rejection comprises primarily an adaptive immune response targeted at destroying the allograft. Central players in this concerted attack are the T lymphocytes, which intervene in the rejection process both by directly killing the foreign cells of the allograft (mainly the cytotoxic T cells), as well as by secreting a plethora of modulatory cytokines (mainly the helper T cells), which will further influence the activity of other immune cells. Generally, Th1 and Th17 cells are considered to drive the rejection of transplanted organs, whereas Th2 cells counteract this. The alloimmune response will also trigger the activation of regulatory T cells, which comprise a subset of T cells, mostly with the CD4+CD25+highfoxp3 phenotype, that are able to steer the immune response towards tolerance. Resident renal cells, such as tubular epithelial cells and endothelial cells, are also active players during acute rejection. They can function as “nonprofessional” antigen-presenting cells and produce pro-inflammatory cytokines and chemokines, hence contributing to the perpetuation of the inflammatory response. Infiltration of CD8+ T cells is considered a specific marker for acute allograft rejection, with the substantial infiltration of CD8+ T cells in the untreated grafts confirming acute allorecognition in our model. Adenoviral transduction of the donor kidney with a construct expressing human IDO significantly decreased the interstitial infiltration of donor kidneys with CD8+ T cells, possibly by reducing their chemotaxy, local proliferation and/or survival the infiltration of CD8+ T cells. Also in rats, ex vivo gene delivery of IDO to the donor heart reduced graft infiltration with inflammatory cells, including CD8+ T cells. The inhibitory effects of IDO on T-cell proliferation and survival have been widely demonstrated in both in vitro and in vivo studies. Specifically, in vitro overexpression of IDO by syngeneic fibroblasts in a coculture with pancreatic islets dramatically reduced lymphocyte proliferation. Furthermore, human vascular endothelial cells transfected with IDO gene were incapable of stimulating allogeneic T-cell responses and induced anergy of allospecific T cells in vitro. Moreover, IDO transfected murine corneal endothelial cells inhibited allogeneic T-cell proliferation.
and prolonged graft survival\textsuperscript{44}. However, adeno-associated IDO gene therapy did not have any effect on graft survival in liver transplantation\textsuperscript{45}. A possible explanation is the different pattern of gene expression in these two studies. In the liver transplantation study, IDO was widely expressed in the liver parenchyma, and the substantial reduction in the circulating levels of tryptophan may have affected the capacity of liver to regenerate after ischemia-reperfusion injury. In the present study, a numerically minor population of cells, mostly interstitial fibroblasts, as previously reported\textsuperscript{24}, express the transgene. The particular expression of IDO in the fibroblasts may be implicated in the specific induction of regulatory T cells in the microenvironment\textsuperscript{43}, without interfering with tryptophan metabolism on a broader scale.

The mechanism of acute rejection involves IL-2 induced T-cell proliferation and differentiation\textsuperscript{46}. We found a remarkable decrement in the IL-2 mRNA levels in the RGDAdTIDO-treated rats, which is in line with recent literature showing decreased IL-2 production by the T cells in the presence of IDO\textsuperscript{47}. In addition, decreased IL-2 production by the resident renal cells may also be involved. Furthermore, down-regulation of IL-17 was found, which is in line with the effects of IDO found in heart transplantation\textsuperscript{48}. Previously, up-regulation of IL-17 was reported in acutely rejected kidneys by infiltrating monocytes\textsuperscript{49}. Because the level of IL-17 reduction was approximately equal to that of the macrophage infiltration, it may well be that the effect on IL-17 is the result of reduced macrophage infiltration only. However, an effect on IL-17 production by the infiltrating cells cannot be excluded.

TGF-β plays a peculiar role in the mechanism of graft rejection. TGF-β is a powerful immunomodulatory molecule, involved in early transplant tolerance\textsuperscript{22}, whereas long-term TGFβ expression is involved in mesenchymal to epithelial transition and fibrosis\textsuperscript{50}. In the present study, decreased TGF-β expression was associated with diminished interstitial a-SMA expression, which is considered a marker of early/pre-fibrosis.

In addition to the well known effects of IDO on T-cell responses, the data obtained in the present study suggest that IDO may also play a regulatory role on the influx of macrophages into the renal interstitium because a decreased number of infiltrating macrophages was found in the RGD-AdTIDO-treated rats. The influx of macrophages is recognized as an important factor involved in allograft dysfunction because macrophages can act as antigen-presenting cells via MHC II molecules, increase immune cell infiltration by secretion of numerous proinflammatory cytokines and chemokines, and dismantle the graft directly\textsuperscript{51}. Although a direct effect of IDO on macrophage function, proliferation and infiltration is yet unknown, a relationship between IDO and macrophage influx was
suggested previously. In a heart transplantation model, decreased infiltration with macrophages was found in the IDO group\textsuperscript{42}. Moreover, in mice with nephrotoxic serum nephritis, treatment with the IDO inhibitor 1-methyl-tryptophan resulted in enhanced infiltration of inflammatory macrophages in glomeruli and tubulointerstitium\textsuperscript{52}. Remarkably, decrease in macrophage and CD8+ cells infiltration occurred in the present study, despite a more robust GFP expression in the RGDAdTIDO group compared to the RGD-AdTL group.

Ischemia/reperfusion injury (IRI) represents an intrinsic problem in kidney transplantation, which impairs early transplant function. Previously, it was shown that IDO gene therapy protects against IRI in lungs\textsuperscript{53}. However, in contrast, early inhibition of IDO protected against kidney IRI in mice\textsuperscript{54}. In our model, we cannot distinguish between the effects of IDO on IRI and acute rejection. It may well be that the immunosuppressive effects of IDO overrule the deleterious effects on IRI. Another explanation for the apparently contradictory effects found in these two studies may be given by the time pattern of gene expression after gene delivery to the kidney. Namely, the transgene comes to expression at approximately 24h after gene delivery, comprising the moment at which most of the IR damage has already taken place.

In the present study, we have employed adenovirus-mediated gene delivery to achieve IDO expression in the renal graft. Previously, we have shown that local delivery of the vector induced strong expression of the transgene in the kidney, whereas only slight expression was found in the liver and spleen\textsuperscript{32}. In the present study, we did not investigate IDO expression in organs other then the kidney; however, systemic levels of kynurenines were not affected by RGD-AdTIDO therapy. Interestingly, immunohistochemical staining demonstrated a 4.5-fold stronger GFP expression in the RGD-AdTIDO compared to RGDAdTL group at day 7, despite renal transduction with equal amounts of viral titers. Previously, we demonstrated that renal transduction of RGD modified adenoviruses declines logarithmically over a period of 2weeks after renal transduction, apparently through cytotoxic T-cell mediated clearance of the transduced cells\textsuperscript{24}. The findings of the present study suggest that the 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. However, we cannot rule out the possibility that IDO influences in vivo the infectivity of the adenovirus (i.e. the initial viral load). This observation brings up the opportunity of adding IDO to other genes within delivery systems that rely on infection, to enhance tissue transduction \textit{in vivo}. 
In conclusion, the present study provides the first evidence for a beneficial effect of adenovirus-mediated IDO therapy on early renal graft damage in an allotransplantation model. Whether the renal graft benefits over the long-term from IDO (gene) therapy remains to be investigated in models of chronic allograft nephropathy.

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