The therapeutic potential of indoleamine 2.3-dioxygenase in kidney transplantation

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Chapter 4

The effects of adenoviral overexpression of Indoleamine 2,3-dioxygenase in bone-marrow derived rat dendritic cells on T cell proliferation and phenotype


submitted
Abstract

Background: Targeting the interaction between antigen presenting cells and T cells represents an appealing tool for acquiring graft-specific tolerance. In this study, we investigated the feasibility of enhancing the expression of Indoleamine 2.3-dioxygenase (IDO) in rat dendritic cells (DC), as a strategy to obtain tolerogenic dendritic cells. The phenotype of these adenoviral-transduced dendritic cells overexpressing IDO was analyzed. Moreover, the effects of transduced DC on the proliferation and the phenotype of the T cells were investigated in vitro.

Methods: DC were obtained from bone marrow of Brown Norway rats. Nine days after harvesting, DC were transduced with an RGD modified adenovirus carrying the gene for human IDO (RGD-AdTIDO) or the gene for luciferase (RGD-AdTL). Untransduced DC served as control. At day 11, DC were used as stimulators in a mixed leukocyte reaction (MLR) with naïve Lewis rat lymphocytes as responders. Phenotype of DC and T-cells was assessed by FACS and real-time PCR.

Results: Both in RGD-AdTIDO and RGD-AdTL transduced cells CD86 membrane expression was decreased in DC compared with the untransduced cells. However, in the MLR only the DC overexpressing IDO suppressed the proliferation of the naïve T cells, which was associated with an up-regulation of foxp3, IL-10, IL-13, IL-2 and IL-17 gene expression.

Conclusion: Although both RGD-AdTL and RGD-AdTIDO transduced DC showed an immature phenotype, only the IDO-overexpressing DC inhibited the proliferation of the naïve T cells in vitro. Moreover, these T cells exhibited a mixture of tolerogenic and pro-inflammatory cytokine profile. Whether the anti- or the pro-inflammatory effects of IDO transduced DC will predominate in vivo remains to be established.
Introduction

Dendritic cells (DC) are highly-specialized antigen presenting cells able to modulate the T-cell reactivity to foreign antigens. While DC are key regulators of the immune outcome, they are capable of both promoting and suppressing T-cell responses, depending on their functional state of maturation. Mature DC possess a strong immunostimulatory capacity, which is reflected by high levels of cell surface class II major histocompatibility complex (MHC II), integrin CD11c and co-stimulatory molecules such as CD80 (B7-1), CD86 (B7-2) and CD40. Consequently, fully mature DC induce a potent, antigen-specific response characterized by the induction of T helper (Th) type 1 CD4+ T cells, cytotoxic CD8+ T cells and the activation of natural killer cells. In contrast, immature DC, which were previously regarded as mere sentinels for antigens, diminish T-cell reactivity and induce tolerance. Immature DC, characterized by a low expression of both MHC II antigens, CD11c integrin and co-stimulatory molecules, promote peripheral tolerance by several mechanisms including T-cell anergy, immune deviation (i.e. shifting from a Th1-dominated response toward a Th2 response and inducing infectious tolerance), T-cell apoptosis and stimulation of regulatory T cell (Treg) formation. Specifically, immature DC can differentiate naïve CD4+ T cells into different Treg classes, including CD4+CD25+foxp3 Treg and type 1 T regulatory cells (Tr1), which inhibit proliferation of Th1 cells. Thus, mature DC induce immunity, while immature DC promote tolerance to the presented antigen.

Because of the central role of DC in modulating T cell reactivity, modification of their function by, for instance, genetic manipulation with tolerogenic molecules, is an attractive approach to treat immune-mediated diseases, including transplant allograft rejection. Indoleamine 2,3-dioxygenase (IDO) is an enzyme that catabolizes the essential aminoacid tryptophan into kynurenines and plays a crucial role in the foeto-maternal tolerance. Tryptophan starvation, accumulation of kynurenines and induction of Treg are considered as the mechanisms underlying the tolerogenic effects of IDO. Overexpression of IDO in solid organ transplantation was previously found to attenuate acute rejection of the lung, heart, islets and skin allograft. We have previously shown that adenovirus-mediated gene therapy with IDO attenuates the features of both acute rejection and CTD in animal models of kidney transplantation. During the last decade, the importance of IDO expression in DC has been recognized. It has been observed that induction of IDO by DC is necessary for the protection from graft versus host disease (GVHD), as INFγ -/- mice succumbed to GVHD very rapidly due to the failure of IDO expression in DC. Moreover, IDO is able to steer the phenotype of DC towards...
tolerogenesis, and after ligation with CTLA-4, immunogenic DC may convert into tolerant, IDO expressing DC\textsuperscript{18}. Therefore, DC genetically modified to express IDO represent an appealing alternative in the treatment of allograft rejection.

Although viral delivery of IDO may be an effective therapeutical tool, viral transduction of DC may be associated with changes in the DC phenotype dependent on both the viral vector as well as the maturation state of the DC\textsuperscript{18,19}. Overexpression of IDO in DC has been studied before\textsuperscript{2,20,21}. Notably, transfection of fully mature DC with IDO using an adenoviral vector suppressed T cell proliferation and induced foxp3 expression, while the DC maintained their mature state\textsuperscript{20}. As a first step towards the use of IDO-overexpressing DC in animal models of kidney transplantation, we sought here to characterize these genetically modified DC and their effects on T cell proliferation and phenotype \textit{in vitro}. 
Material and Methods

Animals
Animals were housed in a light- and temperature-controlled environment and fed standard rodent chow and water ad libitum. Bone marrow (BM) was obtained from Brown Norway (BN, RT1\(^b\)) rats weighing 150 to 175 g. T cells were obtained from BN or Lewis (LW, RT1\(^l\)) rats weighing 150 to 175 g (all from Harlan Laboratories, The Netherlands). Experimental protocols were approved by the Animal Research Ethics Committee of the University of Groningen, the Netherlands.

Isolation and culturing of bone marrow derived dendritic cells
BM-derived dendritic cells (DC) were obtained as previously described\(^{3,4,22}\) with minor modifications. BM cells were cultured in 24-well plates (1x10\(^6\)/well) in 1 mL of RPMI 1640 supplemented with 10% foetal bovine serum (to stimulate full maturation) and 100 U/mL of granulocyte-macrophage colony-stimulating factor (GM-CSF) and 20 ng/mL interleukin (IL)-4 (Insight Biotechnology, Wembley, UK). Every second day, 75% of the medium was replaced with fresh medium containing GM-CSF and IL-4. At day 9, cells were harvested, re-plated at 1x10\(^6\) cells/mL in serum-free RPMI 1640, and transduced with RGD-AdTL or RGD-AdTIDO at multiplicity of infection (MOI) 300 for 3 hours. Subsequently, serum, GM-CSF, and IL-4 were restored and cell culture continued until day 11. At day 11, cells (control-DC, RGD-AdTL-DC and RGD-AdTIDO-DC were harvested, cell surface immunophenotypic analyses was performed by fluorescent-activated cell sorting (FACS Calibur cytometer, San Jose, CA, USA) and data acquisition and analyses was performed using Kaluza 1.2 cytometry software (Beckman Coulter, Inc, Brea, CA, USA). Cells were used further as stimulators for primary mixed lymphocyte reaction. Mouse monoclonal antibodies specific for rat determinants included antibodies specific for CD11c (FITC-conjugated; Serotec), CD86 (PE-conjugated; eBioscience) and class II MHC (OX6, Serotec). FITC-conjugated goat-anti-mouse immunoglobulin G (BD Biosciences) was used as a secondary antibody for the anti-OX6 antibody.

Adenoviral vectors for gene transfer into dendritic cells
RGD-modified adenoviruses\(^{23}\) were used as vectors for gene delivery into the DC. 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 as tracking) 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 (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.

Mixed lymphocyte reaction
Primary mixed lymphocyte reaction (MLR) was performed using transduced BN RGD-AdTL-DC, RGD-AdTIDO-DC or untransduced control-DC and LW lymph node (LN) cells (1x10⁶/well) obtained by passing lymph node suspensions through a stainless sieve. Cultures were maintained in RPMI/foetal bovine serum 20% in 5% carbon dioxide in air at 37°C for 4 days. The DC-to-LN cell ratio was 1:100; BN lymph node cells (1x10⁶/well) were used for the control syngeneic condition. The proliferation response was evaluated by CyQUANT® NF Cell Proliferation Assay kit (Invitrogen Life Technologies, Bleiswijk, the Netherlands) and expressed as the intensity of fluorescence. At the end of MLR, cells (BN RGD-AdTL-DC-, RGD-AdTIDO-DC- and control-DC-generated LW T cells) were collected and used further for PCR analysis.

RNA isolation and real time PCR
To analyze gene expression in T cells stimulated by DCs, RNA was isolated using NucleoSpin RNA/Protein kit (Bioké), which included a DNAsse step. Experiments were performed in triplo. Integrity of RNA was verified 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 human IDO and rat foxp3, IL-2, IL-4, IL-6, IL-10, IL-13, IL-17, TNF-α, TGF-β, and INF-γ gene expression using a real-time PCR protocol, as described previously. Sequence-specific PCR primers (table 1) were purchased from Biolegio (Nijmegen, The Netherlands). All expression values were standardized for GAPDH expression.
### Table 1. Sequences of the primers used in real time PCR analysis of gene expression.

<table>
<thead>
<tr>
<th>gene</th>
<th>forward primer (5':→3')</th>
<th>reverse primer (5':→3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>human IDO</td>
<td>TCATGGAGATGTCCGTAAGG</td>
<td>GCCAAGACACAGTGCATA</td>
</tr>
<tr>
<td>foxp3</td>
<td>CCACACCTCTCTCTTCTCCTT</td>
<td>TGACTAGGGGGCAGTGAAGGC</td>
</tr>
<tr>
<td>IL-2</td>
<td>ATGCAGCTGCATCTGTGTGT</td>
<td>CAATTCTGTGCCTGCTTGAG</td>
</tr>
<tr>
<td>IL-4</td>
<td>TGATGGGTCTCAGCCCCACCTTG</td>
<td>CTTCAGTGTTGTGAGCGTGGACTC</td>
</tr>
<tr>
<td>IL-6</td>
<td>TTCCAGCCAGTTGCCCTTCTT</td>
<td>CAGTGCATACCCGCTGATCA</td>
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<tr>
<td>IL-10</td>
<td>CAGCTGAGCAGCTGTCACTGA</td>
<td>GTCCAGATAGCCGGGTGGT</td>
</tr>
<tr>
<td>IL-13</td>
<td>ATCACAAGAGACAGAAGACTTC</td>
<td>AACTGGGCTACTTGGGATTTT</td>
</tr>
<tr>
<td>IL-17</td>
<td>ATGTGGCCTGATGCTGCTGCTA</td>
<td>TTAGGACGATGGGCGAATAG</td>
</tr>
<tr>
<td>TNF-α</td>
<td>CACGGCTTCTGTACTGTA</td>
<td>GTACCCAGATTGTCGTTGG</td>
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<tr>
<td>TGF-β</td>
<td>ATACGGACTGAGTGGCTGTCT</td>
<td>TGGGACTGATCCATGGG</td>
</tr>
<tr>
<td>INF-γ</td>
<td>ACTGCAAAGGCAACACACTATT</td>
<td>AGGTGCAATCGATGACACT</td>
</tr>
</tbody>
</table>

**Western blotting**

IDO protein expression by the RGD-AdTIDO vector was confirmed in transduced 293 cells by western blot analysis. Briefly, transduced cells were washed three times using phosphate-buffered saline and subsequently lysed in 800 ml of M-PER (mammalian protein extraction reagent, Pierce Biotechnology, Rockford, IL, USA). Protein concentrations were determined using a BioRad protein assay (Bio-Rad, Hercules, CA, USA). An equal amount of protein in sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis sample buffer was separated on 4–20% poly(acrylamide)-SDS gels. After transfer to nitrocellulose membranes (Amersham, Little Chalfont, UK), protein samples were incubated with primary mouse antibody against IDO (Chemicon International, Temecula, CA, USA) and thereafter with a horseradish peroxidase-conjugated rabbit anti-mouse antibody (Dako, Carpinteria, CA, USA). Signals were detected by chemiluminiscence using ECL (Amersham, Eindhoven, 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 metabolism of tryptophan to kynurenines in 293 cells transduced with RGD-AdTIDO (MOI of 50), RGD-AdTL (MOI of 50) and in untransduced cells. Cells were
cultured for 24 hours and the concentration of tryptophan and kynurenines was 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 (propylsulphonic acid) 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.

**Statistical Analyses**

Data are presented as mean ± SEM and significance was tested with one way ANOVA followed by a least significant difference post hoc test. Differences were considered significant at p<0.05.
Results

Expression of a functional human IDO with the RGD-AdTIDO construct

First, the expression of (human) hIDO protein by the RGD-AdTIDO construct was verified in vitro in 293 cells (data replicated from chapter 2). An expected, a 42 kDa protein band was detected in the cells transduced with RGD-AdTIDO, whereas cells transduced with the RGD-AdTL construct or untransduced control cells did not show any IDO expression (Figure 1). Furthermore, functionality of the IDO construct was verified by assessing in vitro the metabolization of tryptophan to kynurenines in 293 cells transduced with RGD-AdTIDO and RGD-AdTL, as well as in untransduced cells. As shown in Table 2, IDO expression by the RGD-AdTIDO both depleted tryptophan content and increased kynurenine concentration in cell lysates compared to controls, confirming the functionality of the RGD-AdTIDO construct.

![Figure 1. IDO protein expression detected by western blotting in 293 cells.](image)

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 (as shown previously).

<table>
<thead>
<tr>
<th></th>
<th>Tryptophan (μmol/l)</th>
<th>Kynurenines (μmol/l)</th>
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<tbody>
<tr>
<td>control</td>
<td>0.48 ± 0.08</td>
<td>&lt;LOD</td>
</tr>
<tr>
<td>RGD-AdTL</td>
<td>0.65 ± 0.17</td>
<td>&lt;LOD</td>
</tr>
<tr>
<td>RGD-AdTIDO</td>
<td>0.12 ± 0.05*</td>
<td>0.92 ± 0.11&quot;</td>
</tr>
</tbody>
</table>

The concentration of tryptophan and kynurenines was determined using HPLC in the 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 (0.001mmol/l). *p<0.05 versus RGD-AdTL and control. †p<0.01 because the value is more than three SDs above the detection limit (as shown previously).
**Expression of hIDO gene by the transduced RGD-AdTIDO-DC**

To confirm hIDO gene delivery with RGD-AdTIDO in DC, IDO expression was assessed using Real time-PCR. IDO mRNA was only found in the RGD-AdTIDO-DC, whereas no hIDO transcript was detected in RGD-AdTL-DC or untransduced control DC (Figure 2).

![Graph showing hIDO/GAPDH expression](image_url)

**Figure 2. Human IDO gene expression in control-DC, RGD-AdTL-DC and RGD-AdTIDO-DC.** On day 11, cells (n=3 experiments/group) were harvested, RNA was isolated and converted to cDNA. Real-time PCR was performed using primers specific for the human (h)IDO. RGD-AdTIDO transduced DC expressed hIDO gene, whereas no expression of hIDO gene was found in DC transduced with RGD-AdTL (RGD-AdTL-DC) or in untransduced (control) DC.

**Transduction with RGD-adenovirus down-regulated CD86 (B7-2) expression**

To determine the maturation state of the DC, we assessed the expression of cell surface class II major histocompatibility complex (MHCII), integrin CD11c and co-stimulatory molecule CD86. In our experiment, BM-derived control DC expressed high levels of CD11c (85.9±6.8%), MHCII (81.7±9.4%) and CD86 (80.2±3%) (Figure 3). Because both RGD-AdTIDO and RGD-AdTL carry the gene for the green fluorescence protein (GFP), it was not possible to assess the expression of CD11c and MHCII in transduced DC, as antibodies for these markers were labeled with fluorescein isothiocyanate (FITC) which show considerable overlap with the fluorescent spectrum of GFP. Therefore, only the red fluorescent labeled antibody for CD86 was used. Interestingly, transduction with either of the RGD-adenoviruses dramatically reduced the expression of CD86 (RGD-AdTIDO-DC...
22.7±6.5%, RGD-AdTL-DC 25.7±6.6%; Figure 4) in comparison with control-DC (80.2±3.0%; p<0.05).

**Figure 3. FACS analyses for CD11c, MHCII and CD86.** Staining was performed with FITC-conjugated anti-CD11c and anti-MHCII mAb, or with PE-conjugated anti-CD86 mAb on control-DC, RGD-AdTL-DC and RGD-AdTIDO-DC. The results are representative of three independent experiments.

**Figure 4. FACS analyses for CD86.** Staining was performed with PE-conjugated anti-CD86 mAb on control-DC, RGD-AdTL-DC and RGD-AdTIDO-DC. Transfection with RGD-AdTL and RGD-AdTIDO markedly reduced the expression of CD86 on the cell surface (*p<0.05 vs control-DC). The results are expressed as percentage of CD86 positive cells.
**RGD-AdTIDO-DC inhibited naïve T-cell response in primary MLR**

To study the influence of RGD-AdTIDO-DC on naïve T cells, we performed MLR. T cells were isolated from LW rats (allogeneic combination) and from BN rats (syngeneic combination). Transduction with both RGD-AdTL and RGD-AdTIDO did not influence the proliferation of syngeneic T cells in comparison with control DC. However, control DC exhibited a potent stimulatory capacity towards allogeneic T cells (p<0.05; Figure 5), as documented by high proliferation, which was unaffected by transduction with RGD-AdTL (Figure 5). In contrast, transduction of DC with RGD-AdTIDO markedly reduced their ability to stimulate naïve allogeneic T cells during MLR (p<0.05; Figure 5).

**Figure 5. IDO expression inhibits primary MLR.** RGD-AdTL-DC or RGD-AdTIDO-DC were used as stimulators in MLR with LN cells from naive LW (allogeneic combination, black bars) or BN rats (syngeneic combination, white bars). Proliferation was expressed as intensity of fluorescence. Control-DC and RGD-AdTL-DC significantly induced the proliferation of LW T cells. RGD-AdTIDO significantly suppressed proliferation of LW T cells. *p<0.05 vs RGD-ADTL-DC and vs control-DC (allogeneic combination).
**RGD-AdTIDO-DC induced Treg, Th2 and Th17 subsets of T helper cells**

Because DC play a pivotal role in the differentiation of naïve CD4 T cells, we evaluated the mRNA expression of a panel of cytokines specific for Th1, Th2, Th17 and Treg cell subsets.

Forkhead box p3 (foxp3), the exclusive product of iTreg, was significantly up-regulated in T cells stimulated with RGD-AdTIDO-DC, compared to T cells stimulated with RGD-AdTL-DC or control-DC (p<0.05, Figure 6A). The immunosuppressive cytokine TGF-β mRNA was up-regulated in RGD-AdTIDO-DC stimulated T cells (p<0.05 in comparison with control-DC; Figure 6B). Furthermore, the expression of IL-10, which is produced by Tr1 subpopulation of Treg and Th2 cells, was also increased in the RGD-AdTIDO-DC stimulated T cells (p<0.05 in comparison with both RGD-AdTL-DC and control-DC; Figure 6C).

The expression of IL-4 mRNA, which is a Th2 cytokine, was significantly elevated in both RGD-AdTIDO-DC and RGD-AdTL-DC stimulated T cells, compared to control-DC treated cells (p<0.05; Figure 7A). IL-13, which is another Th2 type cytokine, was increased significantly only in the RGD-AdTIDO-DC stimulated T cells (p<0.05 vs RGD-AdTL-DC and control-DC; Figure 7B).

The expression of IL-2, which is a Th type 1 cytokine was significantly increased in the T cells stimulated with RGD-AdTIDO-DC in comparison with RGD-AdTL-DC and control-DC treatment (p<0.05; Figure 8A). However, no difference was found in the expression of IFN-gamma (Figure 8B), TNF-α and IL-6 between the groups (data not shown).

Further, higher expression of IL-17, a product of Th17 cells was found in the RGD-AdTIDO-DC group, compared to RGD-AdTL-DC and control-DC groups (p<0.05, Figure 8C).
Figure 7. Analyses of IL-4 and IL-13 mRNA expression. After day 4, cells (n=3) from MLR (allogeneic combination) were harvested, RNA was isolated and converted to cDNA. Real-time PCR was performed. The expression of IL-4 mRNA (A) was upregulated in T cells stimulated with both RGD-AdTL-DC and RGD-AdTIDO-DC; #p<0.05 vs control-DC). The expression of IL-13 mRNA (B) was upregulated in T cells stimulated with RGD-AdTIDO-DC; *p<0.05 vs RGD-ADTL-DC and vs control-DC. Values were standardized on GAPDH.

Figure 6. Expression of foxp3, TGF-β and IL-10 mRNA in T cells co-cultured with control-DC, RGD-AdTL-DC and RGD-AdTIDO-DC. After day 4, cells (n=3 experiments/group) from MLR (allogeneic combination) were harvested, RNA was isolated and converted to cDNA. Real-time PCR was performed. The expression of the foxp3 mRNA (A), TGF-β mRNA (B) and IL-10 mRNA (C) was upregulated in T cells stimulated with RGD-AdTIDO-DC; *p<0.05 vs RGD-ADTL-DC and vs control-DC; # p<0.05 vs control-DC. Values were standardized on GAPDH.
Figure 8. Analyses of IL-2, INF-γ and IL-17 mRNA expression. After day 4, cells (n=3) from MLR (allogeneic combination) were harvested, RNA was isolated and converted to cDNA. RT-PCR was performed. There was significantly upregulated IL-2 mRNA (A) in T cells stimulated with RGD-AdTIDO-DC, no change in INF-γ mRNA expression (B), and significantly upregulated IL-17 mRNA expression (C) in T cells stimulated with RGD-AdTIDO-DC; *p<0.05 vs RGD-ADTL-DC and vs control-DC. Values were standardized on GAPDH.
Discussion

In the current work we sought to investigate the feasibility of producing IDO-overexpressing, rat dendritic cells and to analyze their phenotype and effects on T cell proliferation and differentiation \textit{in vitro}.

We successfully delivered a functional IDO construct into DC, confirmed by real time PCR, and studied the phenotype of these cells. Interestingly, the transduction with both the control adenovirus as well as with the adenovirus carrying the gene for IDO changed the phenotype of the cultured fully mature DC into an immature one. This conclusion is based on the high expression of MHCII, integrin CD11c and co-stimulatory molecule CD86 in control-DC, and a marked decrease in the expression of CD86 in both RGD-AdTL and RGD-AdTIDO transduced DC. However, in a mixed lymphocyte reaction with naïve allogeneic T cells, only RGD-AdTIDO-DC suppressed the proliferation of the T cells, almost to the level of syngeneic control. Moreover, only RGD-AdTIDO-DC influenced the \textit{in vitro} differentiation of naïve T cells into a subpopulation of foxp3 expressing T cells. Additionally, T cells stimulated with RGD-AdTIDO-DC featured increased expression of TGF-β, IL-13 and IL-10 mRNA. Our data suggest therefore that the mere down-regulation of the CD86 on the surface of the DC is not sufficient for functional effects as assessed by the MLR.

The effect of the viral transduction on the DC expression of the co-stimulatory molecule CD86 is an unexpected finding. The viral transduction of DC may be associated with alterations in the phenotype as well as in the function of these cells. In general terms, viral vectors that are successful at transducing DC also activate these cells\textsuperscript{19}. The adenoviral vectors are the most efficient at transducing DC\textsuperscript{19}. Nevertheless, the literature on its effects on the phenotype of the transduced DC is controversial. Funeshima et al.\textsuperscript{20} have not found any change in the phenotype of fully mature XS106 DC which were transduced with a recombinant adenovirus carrying the gene for IDO, while another\textsuperscript{28;29} observed radical maturation of DC after adenoviral transduction, leading to increased proliferation of T cells in MLR. This was reversed completely by adding IL-10 into the medium\textsuperscript{30;31}. In another experiment, transduction of immature DC with adenovirus encoding GFP upregulated co-stimulatory molecules and MHCII, whereas no augmentation of the expression of these molecules was found in mature DC transduced with adenovirus\textsuperscript{19}. Moreover, transduction of both immature and mature DC induced the production of Th1 and inflammatory cytokines\textsuperscript{19}. However, the ability of virally-transduced DC to stimulate T cells in MLR was altered. Immature DC potentiated the proliferation of T cells in MLR, whereas mature DC failed to stimulate T cells in MLR because viral transduction of mature DC resulted in an
increased expression of endogenous IDO. Inhibition of IDO restored the ability of DC to stimulate the MLR\textsuperscript{19}. However, in our study, transduction of DC with both control adenovirus and adenovirus carrying IDO decreased the expression of CD86, while the ability of stimulating T cells in MLR was only significantly inhibited in DC transduced with adenovirus carrying IDO. The transduction of DC with RGD modified adenovirus has not been studied previously.

Therefore we can only speculate which aspect led to the changed phenotype of DC when transduced with both RGD-AdTL and RGD-AdTIDO. The two adenoviruses used by us were identical with the exception of the firefly luciferase (in case of the RGD-AdTL) and human IDO (in case of the RGD-AdTIDO), with the RGD motif being inserted in both of them. However, if the insertion of these genes, or the additional RGD motif in our viral construct initiated the phenotype shift, is not clear. Thus, this important finding needs to be further investigated.

Further we show that transduction of DC with RGD-AdTIDO resulted in a markedly suppressed proliferation of allogeneic T cells and a higher secretion of specific types of cytokines in the MLR. Although suppression of T cell proliferation by DC expressing IDO is in line with previous research\textsuperscript{20,32}, the characterization of T cell subtypes induced by IDO has not been thoroughly studied so far. First, generation of Treg, as documented by the enhanced foxp3 expression was found. Although this increase was relatively small, a small increase in the foxp3 expression is sufficient for promoting the tolerance\textsuperscript{32}. Several subsets of CD4+ T cells with regulatory properties have been described, namely CD4+CD25+ Treg that suppress T-cell response in a cell-to-cell contact dependent manner, and Th3 and Tr1 subsets that exert their suppressive activity through the release of immunosuppressive cytokines\textsuperscript{6}. Transduction with RGD-AdTIDO stimulated the expression of IL-10, which is the typical cytokine expressed by Tr1 Treg\textsuperscript{6}. Also, IL-13 and TGF-β were expressed after IDO transduction, however, in case of TGF-β, also by the control virus transduction. A possible explanation for higher TGF-β expression might be the increased number of T cells in the MLR in the RGD-AdTL group in comparison with the RGD-AdTIDO-DC group. Foxp3, IL-10 and TGF-β however, could be expressed by both Treg/Tr1 subsets and tolerogenic DC. DC can promote a tolerogenic environment through production of suppressive cytokines (such as IL-10, IL-13 and TGF-β)\textsuperscript{33}. These tolerogenic DC can expand immunosuppressive Treg, which function to inhibit effector cell proliferation and activation\textsuperscript{33}. It is hard to distinguish therefore, whether the increased expression of IL-10, IL-13 and TGF-β found in IDO transduced DC originates from DC
itself, or from Treg generated by these DC. The former is favored, as DC expressing IDO also co-express IL-10 and TGF-β2.

The mechanism of the immunosuppressive and tolerogenic effect of IDO includes tryptophan depletion and an increased level of kynurenine33. Three pathways may be involved in producing their immune modulatory effects: T cell anergy, T cell apoptosis and promotion of tolerance via foxp3 expression2. Tryptophan depletion and an increased kynurenine level may explain the decreased T cells proliferation. We also found increased regulatory T cell formation, as reflected by up-regulation of foxp3 selectively in the RGD-AdTIDO-DC stimulated group. Tryptophan starvation can be sensed by cells via activation of GCN2 (general control nonrepressed 2) kinase, which directly binds uncharged tRNAs34. It was found that tryptophan depletion resulted in activation of the GCN2 pathway, the down-regulation of CD3 zeta-chain in CD8+ T cells35 and inhibition of Th17 cell differentiation36. Although GCN2 also contributes to Treg generation in an IDO-high environment35, this hypothesis seems falsified by our observation of increased IL-17, a marker for Th17 cells, in RGD-AdTIDO-DC T cells. The increased IL-17 suggests the generation of proinflammatory Th17 cells. Moreover, also IL-2, another pro-inflammatory cytokine was found increased in the group stimulated with RGD-AdTIDO-DC. It is therefore possible, that although IDO suppressed the T cell proliferation and induced Treg subpopulation, it also stimulated particular inflammatory response.

The second mechanism of immune suppression elicited by IDO is due to the direct effects of the tryptophan metabolites, such as kynurenine on target cells34. In addition to kynurenine, also other IDO metabolites can induce immunosuppression36;37. In the case of CD4+ T cells, IDO metabolites favor expansion of foxp3 Treg35;37. Additionally, we found increased level of IL-4 mRNA, an anti-inflammatory cytokine38, in both RGD-AdTIDO-DC and RGD-AdTL-DC stimulated groups. Because IL-4 is generated by Th2 subpopulation of T cells39, it is thus possible, that also a second small colony of Th2 cells is present in both groups. Th2 cells proliferate from naïve T cells after contact with extracellular pathogens39. It might be therefore possible, that this Th2 subpopulation is generated in response to the viral vector itself.

It should be noted that the adenoviral vectors that we have used in transducting DC are partially immunogenic themselves. Thus, it may be crucial, especially in the context of human DC transduction, to use a viral vector which is devoid of any gene expression, i.e. the third generation “gutless” adenovirus, to avoid the unwanted immunogenic stimulation.

In conclusion, we document here for the first time that mature rat DC isolated from bone marrow changed their phenotype towards an immature one after transduction with
RGD-adenoviruses carrying the gene for hIDO and luciferase, as reflected by the decreased surface of CD86 expression. However, it was only the DC expressing IDO that suppressed the proliferation of naïve T cells in vitro. This was associated with increased expression of foxp3, IL-10, TGF-β and IL-13 by the T cells co-cultured with RGD-AdTIDO-DC, which suggests generation of Tr1 subset of T cells. However, also the pro-inflammatory cytokines IL2, IL17 were up-regulated. Whether the anti-inflammatory/tolerogenic milieu would overwhelm the pro-inflammatory effects of these cytokines remains to be investigated.

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Reference List


