IDO-Competent-DCs Induced by IFN-γ Attenuate Acute Rejection in rat Liver Transplantation

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Abstract

Purpose We established a stable rat model of liver transplantation using Sprague-Dawley rats and Wistar rats in order to investigate the role of the IDO gene in acute rejection after rat liver transplantation.

Methods IDO gene expression and IDO enzyme activity were quantified in liver syngeneic grafts and allografts using microdialysis-HPLC. Liver allografts were evaluated for IDO expression by histopathology. We measured liver function-related biomarkers in liver allografts which were re-infused with untreated or IFN-γ-treated dendritic cells (DCs).

Results We found a significant increase in IDO gene expression and IDO enzyme activity in liver allografts compared the sham and syngeneic graft groups. There was a significant correlation between the number of IDO-positive cells and severity of acute rejection. IDO gene expression and enzyme activity was upregulated in the IFN-γ-treated DC group within 7 days after transplantation compared to the untreated DC group and survival rates were significantly improved.

Conclusions Our results suggested that IDO gene expression correlates with the severity of acute rejection and that IFN-γ-induced IDO-positive DCs may attenuate acute rejection and catalyze local tryptophan metabolism via IDO enzyme expression, leading to immune tolerance after liver transplantation.

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Introduction

Liver transplantation in humans was first described in 1967 [1] and is currently performed to treat acute liver failure and end-stage liver disease. Acute or chronic rejection of the allograft are thought to be modulated by both antigen-dependent and antigen-independent (non-immune) factors that modify fibro-proliferative processes and ultimately disrupt graft function [2]. The effectiveness of the transplantation procedure may also be compromised by the adverse effects of immunosuppressive drug therapies administered to prevent acute rejection. Inducing immune tolerance, has been suggested as an effective way to facilitate survival of the functioning allograft and to reduce or eliminate the need for immunosuppressive agents [3, 4]. Some strategies to induce immune tolerance include 1) myoablative therapy combined with induced donor chimerism and human leukocyte matching before organ transplantation [5], and 2) developing regulatory T cells in vivo or purifying regulatory T cells from the blood of prospective organ recipients, enriching or expanding them in vitro and re-injecting them at the time of transplantation to boost the recipient's immune response [3]. These approaches were shown to prevent allograft rejection and extend the survival of transplanted organs in animals [6] and to correlate with tolerance in humans [7]. Glucocorticoid-induced tumor necrosis factor receptor expression was shown to play a role in regulatory T cell-Kuppfer cell interactions and mediate acute rejection in a rat model of liver transplantation [8].

The generation of regulatory T cells by injecting tolerogenic dendritic cells (DCs) has been investigated by several groups [3, 9, 10] and tolerogenic DC therapy was recently used in transplantation [11]. DCs are bone-marrow-derived antigen-presenting cells that have a recognized tolerogenic capability [12] and maintain both central and peripheral tolerance [11]. A new subgroup of DCs was recently identified that expressed high levels of indoleamine 2,3-dioxygenase (IDO) and increased tryptophan metabolism in local tissue, thereby inhibiting T cell proliferation, inducing apoptosis of activated T cells, and resulting in peripheral immune tolerance [13]. IDO catalyzes tryptophan catabolism via the kynurenin pathway [14]. IDO was shown to modulate the immune response by depleting tryptophan and producing kynurenin metabolites [15]. IDO-competent-DCs where the expression of the IDO gene has been upregulated by cytokines, were reported to regulate the differentiation of naïve CD4+ T cells to FOXP3+ Treg cells and induce activation of functionally quiescent Treg cells [15]. Immune tolerance, mediated by tryptophan metabolism via the IDO pathway, has been suggested to play a role in preventing acute rejection in liver transplant animal models [14]. The IDO gene was shown to be upregulated in DC cells by interferon gamma (IFN-γ) [16], suggesting that regulation of the IDO gene in DC cells could be a novel strategy for inducing immune tolerance after transplantation. However, the mechanisms underlying the immunoregulatory role of IDO in transplantation remains unclear.

In this study, we used microdialysis-HPLC to quantify IDO enzyme activity. We explored the role of IDO gene expression and IDO enzyme activity in acute rejection in rats after liver transplantation. We investigated the correlation between the percentage of IDO-positive cells and severity of acute rejection and evaluated the impact of IFN-γ-treated DCs (IDO-competent DCs) on liver acute rejection. Our results provide insights into the mechanisms underlying immune regulation after liver transplantation and may lead to development of new strategies to treat acute rejection of the transplanted liver.

Methods

Establishment of rat Liver Transplantation Model

This study was approved by the Institutional Animal Ethics Committee of Shanghai Jiao Tong University. We used male 12-week-old Sprague-Dawley (SD) and Wistar rats weighing 200–300 g to establish our liver transplantation model. Our liver transplantation was based on a modification of the "rapid technique" [1] and used the modified cuff method and prevention of abdominal cavity bleeding. The donor liver was harvested as previously described [17].

In the first part of study, the experimental groups were comprised of 1) Sham-operated control group ($n=15$) where blood supply to the liver was blocked for 15 min in SD rats after laparotomy to simulate the anhepatic phase; 2) Syngeneic graft group ($n=15$) where the donor as well as recipients were SD rats; and 3) Allograft group ($n=15$) where the donors were Wistar rats and recipients were SD rats.

Measurement of IDO mRNA

We extracted total RNA from 100 mg of frozen liver tissue using Trizol (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA was synthesized from 2 μg of total RNA in a total volume of 20 μl comprising 0.5 μl RNA polymerase inhibitor (50U/μl), 2 μl random hexamer primer (50pM/μl), and 1 μl of M-MLV reverse transcriptase (200U/μl; Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. PCR was performed using the following primers: IDO (F); 5'-GTA CAT CAC CAT GGC GTA TG-3', IDO (R); 5'-GCT TTC GTC AAG TCT TCA TTG-3' to generate a 740 bp product; GAPDH (F) 5'-ACC ACA GTC CAT GCC ATC AC-3', GAPDH (R); 5'- TCC ACC ACC CTG TTG CTG TA -3' to generate a 440 bp product. The IDO gene was amplified by 40 cycles of 94°C for 30s; 55°C for 1 min and 72°C for 1.5 min. The GAPDH gene was amplified by 29 cycles of 94°C for 30s; 61°C for 45 s and 72°C for 1 min. PCR products were analyzed by agarose gel electrophoresis. PCR primers were synthesized at Shanghai Saibaisheng Gene Technology Co., Ltd. (Shanghai, China).

Measurement of Liver Graft Function After Transplantation

Serum was collected at each time point to determine alanine aminotransferase (ALT) and total bilirubin (TB) levels, using an automatic biochemical analyzer (Olympus AU-800 Automated Chemistry Analyzer, Olympus Inc., Tokyo, Japan).

Measurement of IDO Enzyme Activity by Monitoring Tryptophan (Trp) and Kynurenine (Kyn) Levels Using Microdialysis HPLC

IDO enzyme activity is measured by determining local tryptophan and kynurenine concentrations using microdialysis-HPLC, since IDO enzyme is the rate-limiting enzyme of the tryptophan metabolism, and kynurenine is its metabolite.

Rats were implanted with a CMA 20microdialysis probe (CMA20, Stockholm, Sweden) at postoperative day 1 to measure tryptophan and kynurenine concentrations for 14 consecutive days, using standard protocols [18]. Dialysis fluid (lactated Ringer's solution containing 30u/ml heparin (Sigma, USA) was infused at a flow rate of 0.1 ml/h and

dialysis specimens were collected every 3 h and stored at -80°C. Tryptophan and kynurenin concentrations were measured simultaneously in 50 μl of liver dialysis specimens using HPLC (Hewlett Packard Agilent 1100 HPLC System, Agilent Technologies, CA, USA). Tryptophan was measured at excitation wavelength of 285 nm and emission wavelength of 365 nm. Kynurenin and 3-nitrotyrosine were measured at 360 nm. The mean value of 3 readings on the same day was taken as the final result. Data were analyzed using the HP ChemStation for HPLC.

The probe was placed in the standard Trp or Kyn fluid for dialysis each time before implantation of the microdialysis probe. After 2 h of system balancing, dialysis specimens were collected and HPLC testing was performed 3 h later. Trp or Kyn concentrations were determined based on the peak area, and dialysance was calculated by the following formula: Dialysance = concentration of the dialysis specimen / concentration of the standard specimen × 100 %.

Immunohistochemical Evaluation of IDO Expression in Liver Transplants

Liver transplantation was performed as described above using sham operated group ($n=10$) and allogenic strain transplantation group ($n=30$) where the donors were Wistar rats and recipients were SD rats. Acute rejection was pathologically graded on postoperative day 7 and IDO expression was evaluated by immunohistochemistry using standard protocols. Briefly, experimental animals were sacrificed at postoperative day 7 and liver specimens were harvested, fixed with paraformaldehyde for 24 h, embedded with paraffin and pathological sections were made using standard protocols. After routine dewaxing and antigen retrieval, sections were blocked with bovine serum albumin and incubated overnight at 4°C with a 1:1000 dilution of mouse anti-rat IDO mAb (kind gift from Prof. G.C. Prendergast, Thomas Jefferson University, USA). The sections were washed and incubated at 37°C for 30 min with a 1:5000 dilution of anti-mouse-HRP secondary antibody (Santa Cruz Biotechnology, USA). Color was developed with 3, 3'diaminobenzidine (DAB; Sigma) and sections were counterstained with hematoxylin. Primary antibody was replaced with PBS for the negative control. Cells with brown, cytoplasmic staining were defined as IDO-positive and were counted at ×400 magnification in 10 different portal areas.

Pathological Grading of Liver Acute Rejection

Acute rejection of the allograft was classified as mild (level I), moderate (level II) or severe (level III) based on the severity of inflammatory cell infiltration in portal areas, phlebitis, bile duct injury and lobular lesions. We also used the Banff schema for more detailed grading [19] as follows: Level 0: Only mild inflammatory cell infiltration observed in the portal area without other lesions. Level I: Inflammatory cell infiltration exists only in a few parts of the portal area. Level II: Inflammatory cell infiltration involves most or all parts of the portal area. Level III: Inflammatory cell infiltration is also observed in the periportal areas, with moderate to severe periphlebitis and inflammatory cell infiltration inside the liver parenchyma and liver cell necrosis around the veins.

Re-Infusion of IFN-γ-Treated DCs into Transplanted Animals

Experimental animals were divided into the DC control group ($n=21$) and the IFN-γ-treated DC group ($n=21$). At day 2, 5, 7, and 14, two rats from each group were sacrificed for collection of liver and blood samples. Additional 3 rats per group were subjected to the microdialysis assay. The rest 10 rats per group were monitored for survival.

Spleens of the donor Wistar rats were harvested by aseptic surgery 6 days before the transplantation and DCs were isolated and cultured using a DC isolation kit (Qikang Biotech Co. Ltd., Shanghai, China). Briefly, 3×10^6 cells were cultured in serum-free DMEM medium containing 50 IU/ml penicillin, 50 μg/ml streptomycin, 1 % MEM and 10 mM HEPES for 2 h at 37°C before replacing the medium with fresh DMEM medium containing 10 % FCS (Hangzhou Sijiqing Biological Engineering Materials Co., Ltd., Hangzhou, China). After overnight incubation, cells were cultured in the presence of GM-CSF (100 ng/ml) and IL-4 (50 ng/ml) for 3 days. TNFα (10 ng/ml) was added on the 4th day. Recombinant rat IFN-γ (100U/ml) was added to the IFN-γ-DC group, but not the control DC group on the 5th day of culture. All culture media, antibiotics, and cytokines were purchased from Life Technologies. Cells were cultured at 37°C for 48 h and washed with serum-free medium.

Liver transplantation was performed using Wistar rats as donors and SD rats as recipients. In the control DC group, DCs that had been cultured for 7 days were re-infused into the recipient liver through portal vein injection on postoperative day 1 (1×10^6 cells/ml). In the IFN-γ-DC treatment group, IFN-γ-treated DCs were re-infused into the recipient animals via the portal vein as described above. DCs were re-infused on postoperative day 1 rather than on day 0 because of the significant increase in mortality seen when the re-infusion was performed on the same day as the transplant (data not shown).

We measured ALT and TB levels in the DC control group and the IFN-γ-DC treatment groups as described above. We also used ELISA kits (Abcam, MA, USA) to determine the blood levels of IL4, IL10, and IFN-γ.

Evaluation of Apoptosis in Liver Allografts of Using TUNEL Assays

Liver allografts were examined for apoptosis 7 days after transplantation between strains using the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) kit (Roche Diagnostics, Indianapolis, IN, USA), according to the manufacturer's instructions.

Statistical Analysis

Data were expressed as mean and standard deviation (mean ± SD). We used t-test or ANOVA to compare differences followed by two-by-two comparisons adjusted with Bonferroni's correction. Data were analyzed using SAS 9.0 software (SAS Institute Inc., Cary, NC, USA.). All P values were two-sided and were considered significant if P was less than 0.05.

Results

IDO Expression is Induced by Liver Transplantation due to Acute Rejection

We established a model of rat liver transplantation using the modified fast method which simplified the surgical process, improved the success rate of the surgery and the long-term survival rate. The mean donor operation time was 21.6 ± 1.95 min and the mean donor liver trimming time was 23.1 ± 1.61 min. The mean recipient operation time was 48.1 ± 2.63 min, the mean anhepatic phase was 15.9 ± 2.26 min, and the ratio of successful surgery was 94.1 % (207/220). Rats with congenic strain transplantation (SD→SD) achieved long-term survival, with a 3-month survival rate of 80.7 %. Various degrees of acute rejection occurred in rats with allogenic strain transplantation (Wistar→SD) at the postoperative day 7, and these rats died from rejection-induced liver failure. The mean survival period in this group was 14.5 days.

We looked at the temporal profiles of ALT, TB, Trp, Kyn and IDO mRNA in the different experimental groups. ALT and TB are important markers of liver function and are upregulated with transplant rejection. There was a significant decrease in serum ALT levels over time in the congenic-strain transplantation group while there was a significant increase in serum ALT levels in the allogenic-strain transplantation group (Fig. 1a). Although total bilirubin levels increased in both groups after postoperative day 2, the magnitude of increase was greater in the allogeneic group (Fig. 1b). Our HPLC results showed significantly lower Trp levels in both transplantation groups compared to the sham-operated group at days 5 and 7 (Fig. 1c). In contrast, Kyn levels in both transplantation groups increased over the study period and were significantly higher than the sham-operated group (Fig. 1d). IDO mRNA levels were
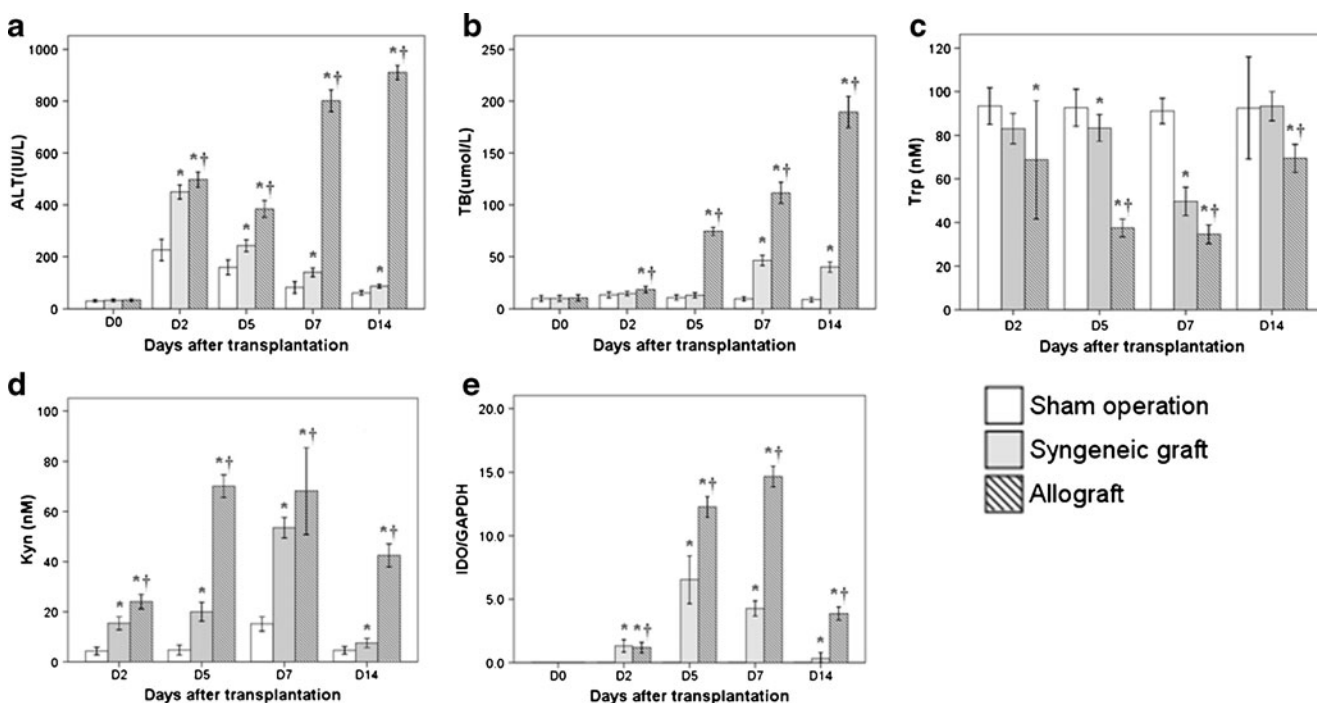

Fig. 1 Temporal profiles of (a) ALT, (b) TB, (c) Trp, (d) Kyn, and (e) IDO mRNA expression in the sham operation, the syngeneic liver graft,, and liver allograft groups. The data were presented as mean ± SD; $n=3$ for each time point, * $p<0.05$ compared to sham-operated group at each time point, † $p<0.05$ compared to same strain transplantation group

significantly higher in both transplantation groups compared to the sham-operated group (Fig. 1e).

IDO Expression is Related to Severity of Acute Rejection

We used immunohistochemistry to determine the expression of IDO in the liver in control rats and in 30 rats which received liver allografts, at 7 days after transplantation. After sacrifice, we found that 15 rats exhibited Banff level I acute rejection, 6 rats exhibited Banff level II acute rejection and 9 rats exhibited Banff level III acute rejection. The control rats all exhibited Banff level 0. We evaluated the correlation between the percentage of IDO-positive cells and severity of acute rejection after transplantation. IDO expression was characterized by brown, cytoplasmic staining (Fig. 2). We showed that IDO-positive cells were mainly distributed in the portal area after transplantation and their morphology was similar to that of monocytes. We showed a positive correlation between the number of IDO-positive cells and aggravation of rejection with varying levels of inflammatory cell infiltration in portal areas (Fig. 2 a-h). Severe rejection was accompanied by invasion of liver parenchyma with the inflammatory infiltration, accompanied by cholangitis and endophlebitis. We showed by immunohistochemistry that there was a correlation between the number of IDO-positive cells in the portal area and severity of rejection (Fig. 2 e-g). We also showed a correlation between the number of IDO-positive cells and the Banff levels. Specimens scored at Banff levels 2 and 3 had a significantly higher number of IDO-positive cells compared to specimens at Banff levels 0 and 1 (Fig. 2i).

IDO-Competent-DCs Treated by IFN-γ in Vitro Plays a Role in Induction of Immune Tolerance

We cultured DCs for 1, 3, 5 or 7 days and treated the cultures with IFN-γ from day 5 to day 7, in order to upregulate the expression of the IDO gene in these cells. We used RT-PCR to evaluate IDO mRNA levels in IFN-γ-treated cells. We showed that IDO mRNA levels were upregulated by day 7 in IFN-γ-treated cells (Fig. 3). Re-infusing IFN-γ –treated DCs into allogeneic liver transplant recipients resulted in a significant decrease in acute rejection symptoms when compared to animals that were re-infused with untreated, control DCs (Fig. 4). We determined the temporal profiles of ALT, TB, Trp, Kyn, IDO mRNA, IL-4, IL-10, and IFN-γ levels in rats re-infused with DCs or IFN-γ-DC groups. Re-infusion of IFN-γ –treated DCs into recipient rats resulted in a significant decrease in ALT and TB levels compared to animals re-infused with control DCs, which exhibited progressive aggravation of liver function (Fig. 4a, b).

We also showed a decrease in Trp levels in the early stages post transplantation and then an increase after day 7 (Fig. 4c). However, Trp levels were significantly higher in the IFN-γ –treated DC group in day 14 compared to the control DC group (Fig. 4c). In addition, our HPLC results showed an increase in Kyn levels in the control DC group reaching a peak at postoperative day 7. Kyn levels remained high until day 14. Kyn levels in the IFN-γ –treated DC group were significantly lower on day 14 compared to the control DC group (Fig. 4d).

Re-infusion with IFN-γ–treated DCs also resulted in a significant upregulation of IDO mRNA levels at the early stages after transplantation (peaking at day 5) compared to re-infusion with untreated DCs where IDO mRNA levels were relatively low at the early stages after transplantation, but gradually increased as a function of aggravated acute rejection (Fig. 4e). It is important to note that IDO mRNA levels in this case were derived from grafted liver tissue, where IDO may not be completely DC-derived. Furthermore, we showed a relative increase in the serum level of IL10 in animals re-infused with IFN-γ –treated DCs compared to animals re-infused with control DCs (Fig. 4g) and a relative decrease in the serum levels of IFN-γ (Fig. 4h).

Evaluation of the Effect of IFN-γ–Treated DCs on Acute Rejection After Liver Transplantation Between Strains Using Histology

We harvested liver tissue from the untreated DC and IFN-γ–treated DC groups on day 2 and day 7 after liver transplantation and performed HE staining in order to evaluate the effect of IFN-γ–treated DCs on acute rejection after transplantation. Liver tissue from the untreated DC group, exhibited infiltration of inflammatory cells in the portal area and bile duct epithelial degeneration on day 2 after transplantation (Fig. 5 a,b). On day 7, the portal area was significantly widened and infiltration of inflammatory cells exceeded the portal area. The lobular structure was damaged, and there was cholestasis and bile duct epithelial degeneration. We observed necrosis as well as proliferation, and endothelial inflammation involving small vessels. We observed Grade II to III rejection in these tissues (Fig. 5 c,d).

Tissue harvested from the IFN-γ-DC group on day 2 after liver transplantation exhibited normal liver structure with a few inflammatory cells in the portal area (Fig. 6a, b). On day 7, the structure of the hepatic lobule was complete and infiltration of inflammatory cells was significant and limited to the portal area. There was no bile duct epithelial necrosis, proliferation or endothelial inflammation involving small vessels. We observed Grade 0 to I rejection in these tissues (Fig. 6c,d).

We used immunohistochemistry to evaluate the expression of IDO after liver transplantation in the untreated DC group (Fig. 7a,b,c,d) and the IFN-γ-DC group (Fig. 7 e,f,g,h) on days
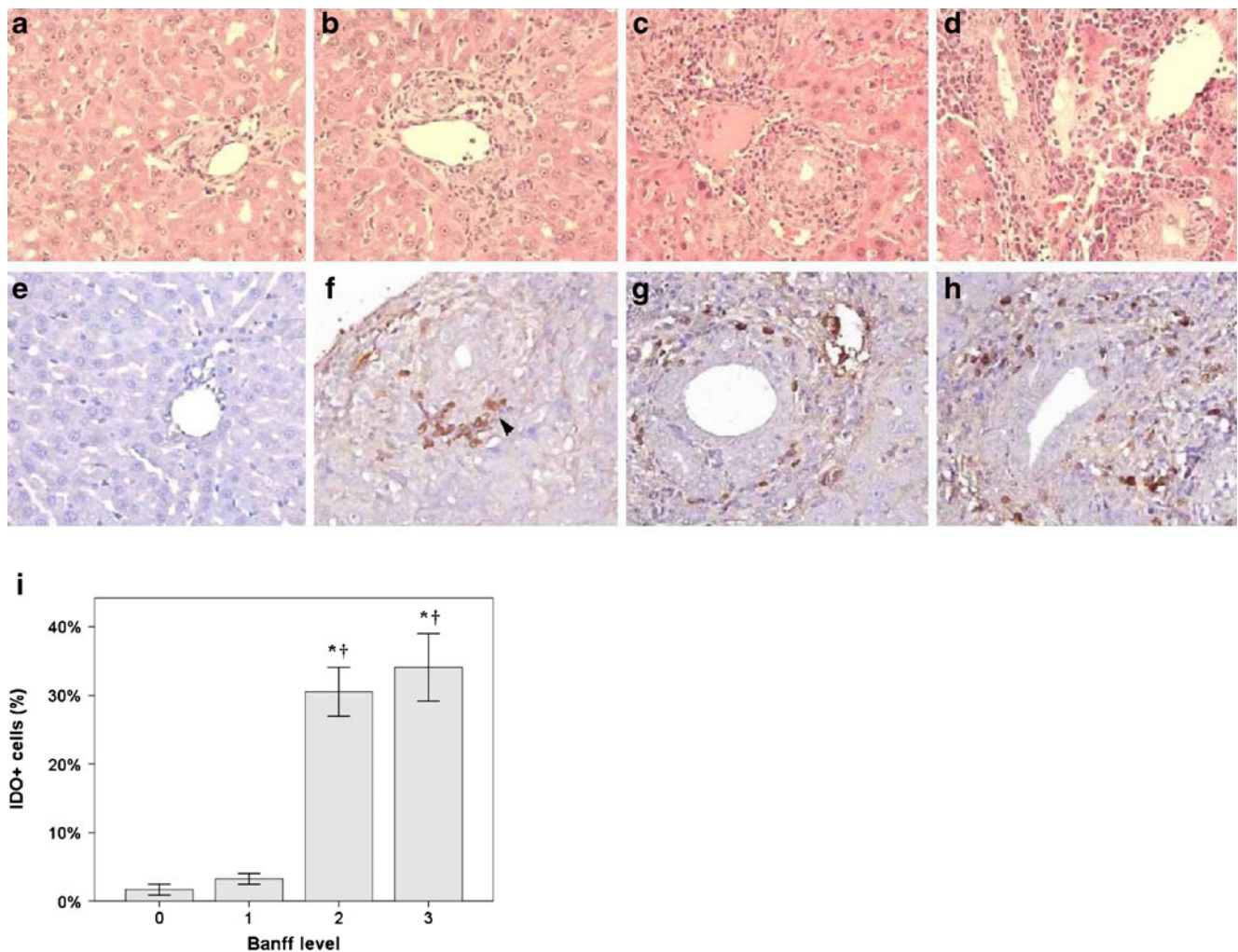
Fig. 2 Correlation between percentage of IDO-positive cells and acute rejection in the sham-operated group (a and e) and liver allograft group with Banff level I acute rejection (b and f), Banff level II (c and g), and Banff level III (d and h). Representative pictures of hematoxylin and eosin staining (A to D) and IDO immunohistochemistry (E to H)

(×400). Correlation between the percentage of IDO-positive cells and Banff levels (i). The mean ratio of IDO-positive cells in 10 different portal areas at various Banff levels was calculated. The data were presented as mean ± SD; $n=10$, * $p<0.05$ compared to Banff level 0, † $p<0.05$ compared to Banff level 1

2, 5, 7, 14 after liver transplantation. Our immunohistochemistry data were consistent with the mRNA data and showed that IDO protein levels were upregulated early after transplantation in the IFN-γ–treated DC group whereas tissues harvested from the untreated DC group exhibited a more gradual increase in IDO levels, which correlated with acute rejection.
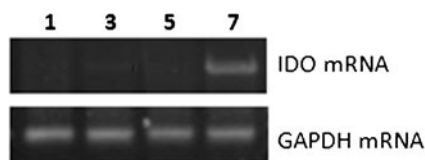
We also used TUNEL assays to evaluate apoptosis of lymphocytes in liver allografts from the control DC group and the IFN-γ-DC group at 7 days after liver transplantation (Fig. 8). Liver allografts from the IFN-γ-DC group exhibited a higher number of apoptotic lymphocytes in the portal area compared to the control DC group (Fig. 8).

Evaluation of the Effect of IFN-γ–Treated DCs on Survival After Liver Transplantation Between Strains

Figure 9 showed that the IFN-γ-treated DC group had a cumulative survival rate of 89 % within 2 weeks of transplantation, and a median survival time of 27 days. In contrast, the control DC group had a cumulative survival rate of 25 % within 2 weeks of transplantation and a median survival time of 12 days. Statistical analysis showed the survival time in the IFN-γ-treated DC
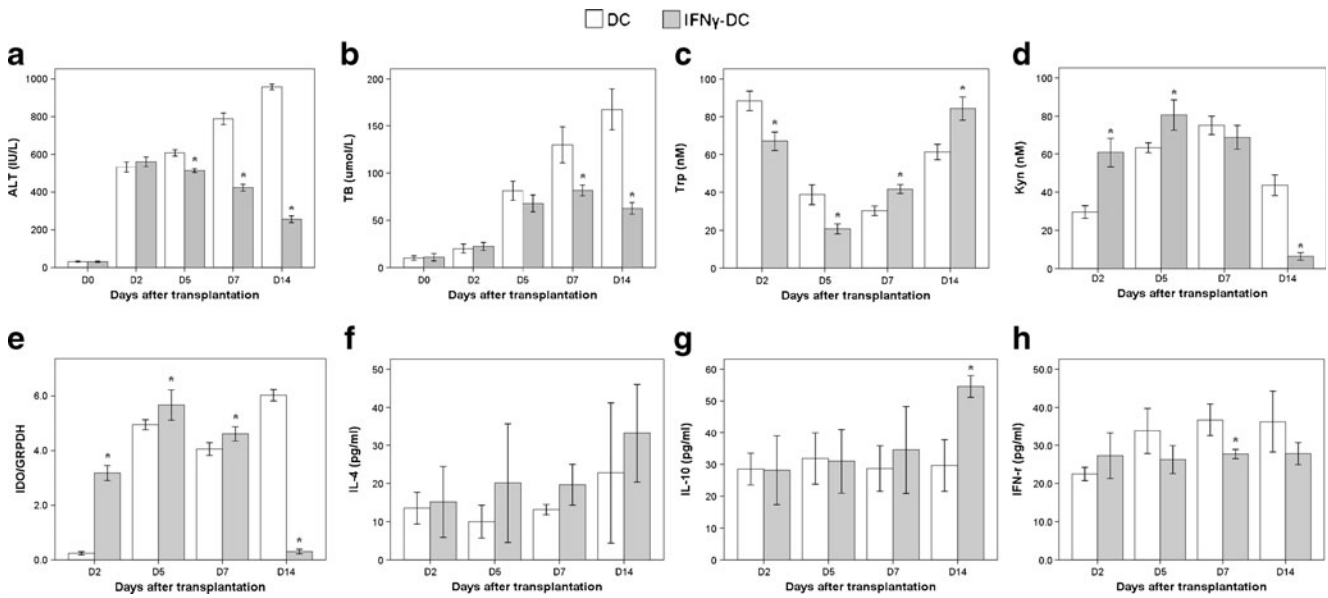

Fig. 3 RT-PCR to evaluate IDO mRNA levels in DCs. IFN-γ induced IDO mRNA expression in DCs. DCs were cultured for various days (1, 3, 5, and 7), and were subjected to RT-PCR to detect IDO mRNA levels. IFN-γ was added into the cultures from day 5 to day 7

□ DC   ■ IFNγ-DC

Fig. 4 Effect of in-vitro IFN-γ-induced DCs on the treatment of acute rejection after rat liver transplantation between strains. Temporal profiles of (**a**) ALT, (**b**) TB, (**c**) Trp, (**d**) Kyn, (**e**) IDO mRNA expression, (**f**) IL-4, (**g**) IL-10, and (**h**) IFN-γ in the DC group and IFN-γ-DC groups. The data were presented as mean ± SD; $n=4$ for each time point, * $p<0.05$ compared to sham-operated group at each time point

group was significantly longer than that in the DC group (Fig. 9).

Discussion

In this study, we established a stable rat model of in situ liver transplantation in order to investigate the relationship between IDO gene expression and post-transplant immune status following liver transplantation. We also evaluated the therapeutic value of using of DCs with IFN-γ-induced IDO expression in the treatment of acute rejection after rat liver transplantation. We showed that 1) IDO gene expression and enzyme activity were significantly upregulated early after rat liver transplantation and decreased gradually along with the controlled acute rejection, suggesting that IDO may play an immunomodulatory role after liver transplantation and 2) there was a significant correlation between

Fig. 5 HE staining to show histology of liver tissue in the control DC group after liver transplantation between strains. **a**, **b**: Day 2 (D2) liver. Significant infiltration of inflammatory cell infiltration in the portal area (×100) and bile duct epithelial degeneration was observed (×400). **c**, **d**: Day 7 (D7) liver. The range of portal area was significantly widened, the inflammatory cell infiltration exceeded the portal area, the lobular structure was damaged, and there was cholestasis (×100). There was degeneration of bile duct epithelial cells, coexistence of necrosis and proliferation, and endothelial inflammation involving small vessels (×400). Grade II to III rejection was observed
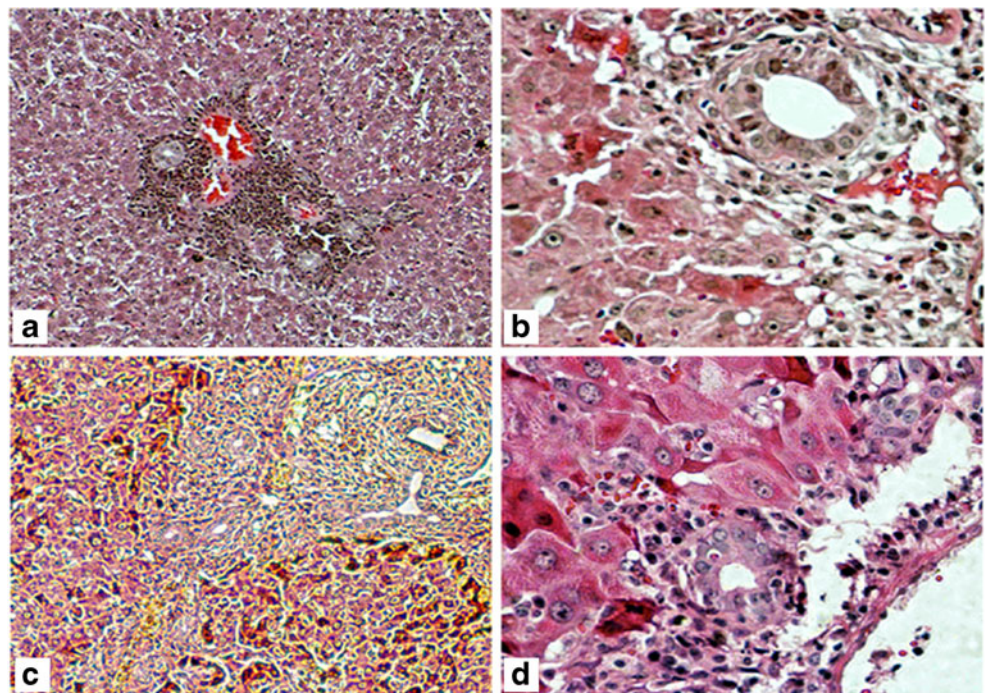
Fig. 6 HE staining to show histology of liver tissue in IFN-γ-DC group after liver transplantation between strains. **a**, **b**: D2 liver. The structure of liver tissue was basically normal (100X), with a few inflammatory cells in the portal area (×400). **c**, **d**: D7 liver. The infiltration of inflammatory cells was limited to the portal area, and the structure of hepatic lobule was complete (×100). Inflammatory cell infiltration was significant in the portal area, but there was no bile duct epithelial necrosis, proliferation and endothelial inflammation involving small vessels (×400). Grade 0 to I rejection was observed
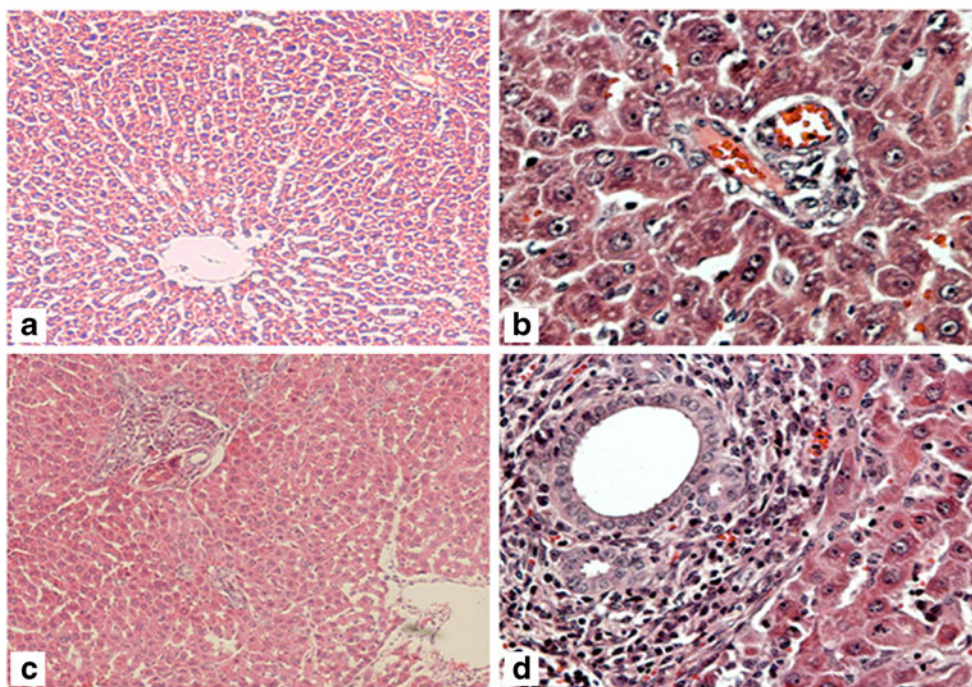
the number of IDO-positive cells and severity of rejection based on the Banff level, suggesting that IDO-positive DCs participate in post-transplant immune regulation. Based on parameters such as recovery of liver function and decreased periportal inflammatory cell infiltration, we showed that the IFN-γ-treated DC re-infused group exhibited significantly decreased symptoms of acute rejection compared to the control DC group. Importantly, we showed increased apoptosis of infiltrating lymphocytes in the IFN-γ-DC group and a significantly higher survival rate compared to the control DC group. Our data suggest that IDO gene expression in DCs plays a role in attenuating rejection and inducing immune tolerance after liver transplantation.

Previous studies of IDO expression suggested that IDO activity mediated long-term organ acceptance post- transplantation [20–22]. IDO-transduced donor-specific DCs induced skin allograft tolerance in an antigen-dependent manner in mouse recipients, suggesting that IDO played a role in DC-mediated allograft tolerance [20]. IDO activity was also thought to play a role in liver-induced long-term acceptance of a kidney graft in a combined liver and kidney transplantation model [21]. However, the molecular mechanisms by which DC-mediated IDO activity induces tolerance were previously only studied in-vitro. Moreover, since the IDO enzyme can exist in the active and inactive states in vivo, determination of IDO gene expression by itself does not provide an accurate picture of IDO activity. We therefore used microdialysis to perform long-term dynamic monitoring of extracellular Trp and Kyn levels and determined IDO enzyme activity in vivo for the first time. The major advantages of our technique are minimal invasiveness, strong real-

time performance, high sensitivity, and good reproducibility. We suggest that the liver microdialysis-HPLC analysis technique is a valuable tool to accurately evaluate the relationship between IDO and immune tolerance.

IDO has been shown to reduce immune rejection and induce immune tolerance via degradation of local tryptophan and generation of kynurenine, and mediating T cell apoptosis [23–25]. IDO may also facilitate the transformation of naïve T cells into Treg cells and subsequent maintenance of Treg cell activity. We suggest that upregulation of post-transplantation IDO levels in our study, may be due to the fact that a large amount of IFN-γ is released following immune rejection, which could play a role in induction of IDO synthesis in DCs, We suggest that IDO promotes the degradation of tryptophan and generation of kynurenine, mediating immune tolerance. Alleviation of immune rejection in animals re-infused with IFN-γ-treated DCs could be closely related to the high expression of IDO in these cells.

IDO-expressing DCs were shown to play a role in regulation of cytokines that favored differentiation of CD4+ T cells in regulatory T cells (Tregs) instead of CD4+ effector T cells; this was thought to be the result of forced IDO expression in DCs, which have a greater capability to expand Tregs [20]. Donor-derived DCs were thought to reduce acute allograft rejection, but could not prevent the infiltration of inflammatory cells and fibrosis in the graft [9]. Donor and F1-derived DCs acted synergistically to induce immune tolerance and inhibit both acute and chronic transplant rejection in mice [9]. To the best of our knowledge, our present study is the first to identify a specific type of IDO-positive cells after liver transplantation, which were mostly

Fig. 7 Immunohistochemical staining of IDO antibody after liver transplantation between strains. **a**, **b**, **c**, **d** are representative images of anti-IDO antibody staining in the liver allografts at D2, D5, D7, D14 respectively in the DC group (×400); **e**, **f**, **g**, **h** are representative images of anti-IDO antibody staining in liver allografts at D2, D5, D7, D14 respectively in the IFN-γ-DC group (×400). Cells with brown cytoplasmic stain were IDO⁺ (red arrows)
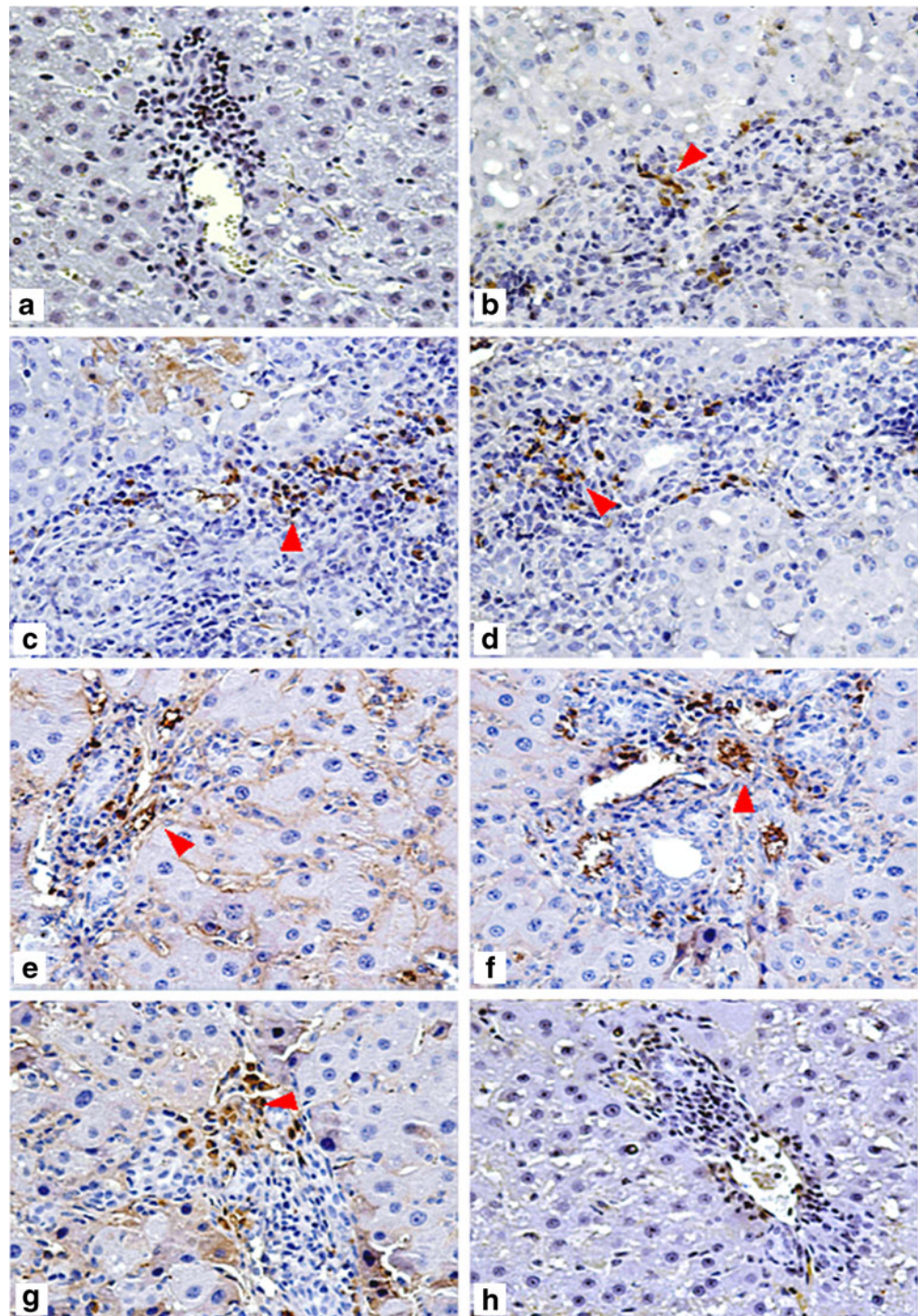
distributed in the portal area and were similar in morphology to monocytes. Our data showing a correlation between increased number of IDO-positive cells in the portal area and severity of rejection, suggested that acute rejection induced the appearance of IDO-positive cells inside the liver after liver transplantation. Although IFN-γ also induced IDO gene expression in donor DCs in vitro, it is not clear if the immune tolerance induced by IFN-γ-treated DCs was due to IDO gene expression. Importantly, we found

increased apoptosis of infiltrating lymphocytes in transplanted animals re-infused with IDO-competent DCs. Importantly, the IFN-γ-treated DC-group showed significantly higher survival rates compared to the control DC-treated group. The advantage of inducing IDO expression in vitro before injecting DCs into the subject rats is that the in vivo physiology of the recipient animals is not disturbed by direct injection of IFN-γ. Our data suggested that increased expression of IDO could originate from liver cells as well as IDO-positive DC

Fig. 8 TUNEL assay to evaluate apoptosis of lymphocytes in liver allografts 7 days after liver transplantation. **a**, Control DC group (100X). A few apoptotic lymphocytes were observed in the portal area. **b**, Control DC group (400X). Infiltration is more obvious with a few apoptotic cells. **c**, IFN-γ-DC group (100X). More apoptotic lymphocytes observed in the portal area compared to the control DC group. **d**, IFN-γ-DC group (400X). More infiltrated lymphocytes undergo apoptosis compared to the control DC group; cell nuclei are dark purple (400X)
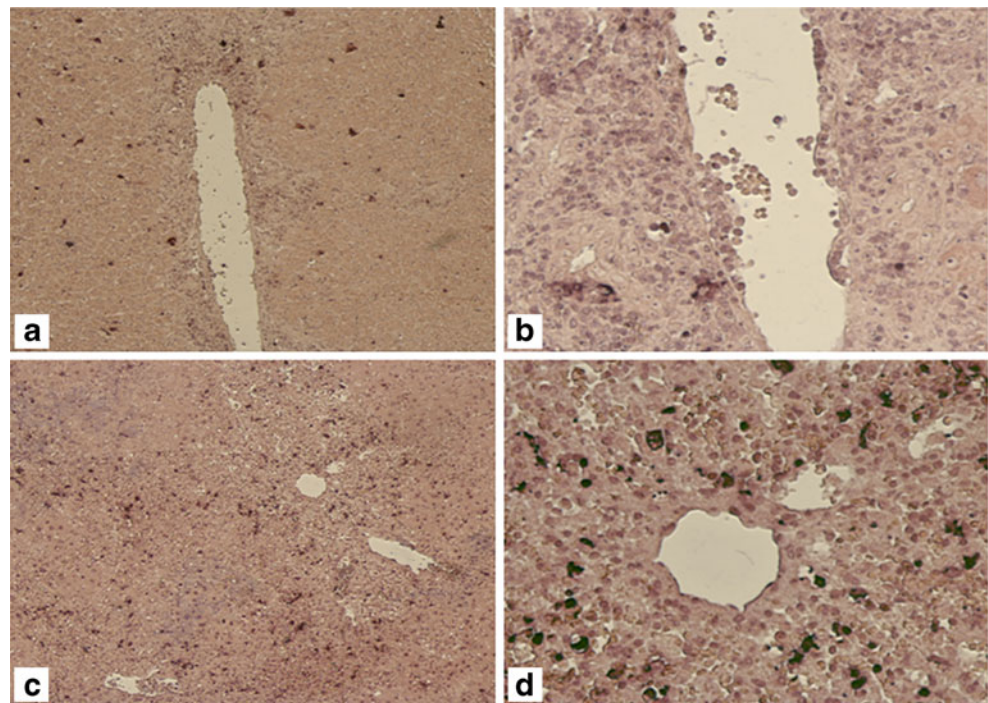
cells moving from the spleen to the liver. Importantly, our results demonstrated that experimental graft rejection can be moderated by the transfer of tolerogenic DC and resultant IDO expression, reinforcing the idea of the therapeutic potential of IDO. It is also interesting to note that ALT levels were higher and signs of immune rejection based on Banff levels were higher in the control-DC-treated animals compared to the allograft group. We speculate that this could be because the DCs may act as antigen presenting cells, which could activate T cells following the transplant procedure, resulting in earlier and more severe rejection compared to the non-DC-treated allografts.
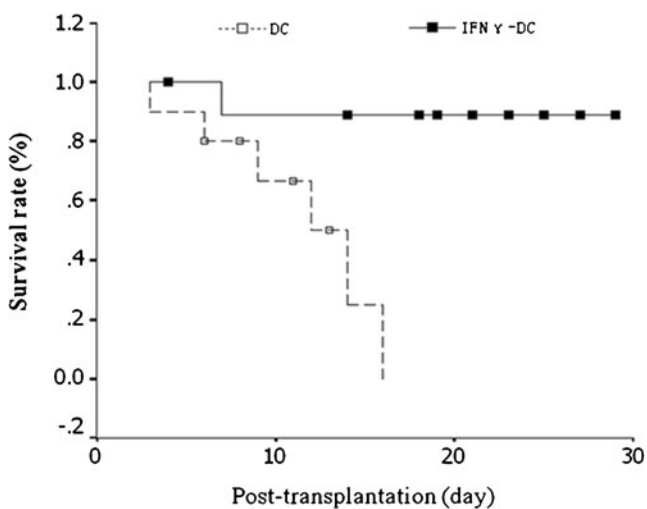

Fig. 9 Survival curves of rats re-infused with untreated DCs and IFN-γ-treated DCs. Rats re-infused with IFN-γ-treated DCs showed significantly prolonged survival rates compared to rats re-infused with untreated DCs

Our study was based on a previous study which showed that IDO-competent-DCs inhibited T cell proliferation, suggesting the influence of tolerance-inducing DCs on T cells [26]. IDO has also been shown to promote the differentiation of CD4 + T cells into Foxp3 + Treg cells, while activating Treg cells and inhibiting the transformation of these cells into T helper cells [15]. However, we did not directly explore the effects of IDO-competent DCs on T cells in vitro, which is a limitation of this study. A second major limitation of this study was that there was no direct down regulation of IDO. It will be interesting to directly assess the role of IDO in our system using IDO knockouts. Additionally, IFN-γ has a broad effect on the immune system making it difficult to directly attribute the effect of interferon on IDO expression. Also, the harvesting and cultivating of donor DC cells is difficult in humans and the technique of inducing DC cells for long-term expression of the IDO gene should be improved.

Conclusions

Our results suggest that IDO enzyme activity plays a role in immune regulation and IDO gene expression correlates with the severity of acute rejection. DCs with IFN-γ-induced IDO expression may also play a role in attenuating acute rejection possibly via IDO-mediated tryptophan metabolism, leading to liver transplant immune tolerance by Th1/Th2 cytokine-induced immune deviation. The results of this study imply that IDO has therapeutic potential in anti-rejection and immune tolerance after transplantation.

References

1. Starzl TE, Marchioro TL, Vonkaulla KN, Hermann G, Brittain RS, Waddell WR. Homotransplantation of the liver in humans. Surg Gynecol Obstet. 1963;117:659–76.

2. Seetharam A, Tiriveedhi V, Mohanakumar T. Alloimmunity and autoimmunity in chronic rejection. Curr Opin Organ Transplant. 2010;15(4):531–6. doi:10.1097/MOT.0b013e32833b31f4.

3. Lombardi G, Sagoo P, Scotta C, Fazekasova H, Smyth L, Tsang J, et al. Cell therapy to promote transplantation tolerance: a winning strategy? Immunotherapy. 2011;3(4 Suppl):28–31. doi:10.2217/imt.11.42.

4. Dresske B, Lin X, Huang DS, Zhou X, Fandrich F. Spontaneous tolerance: experience with the rat liver transplant model. Hum Immunol. 2002;63(10):853–61.

5. Blaha P, Bigenzahn S, Koporc Z, Sykes M, Muehlbacher F, Wekerle T. Short-term immunosuppression facilitates induction of mixed chimerism and tolerance after bone marrow transplantation without cytoreductive conditioning. Transplantation. 2005;80(2):237–43.

6. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. Science. 2003;299(5609):1057–61. doi:10.1126/science.1079490.

7. Taflin C, Nochy D, Hill G, Frouget T, Rioux N, Verine J, et al. Regulatory T cells in kidney allograft infiltrates correlate with initial inflammation and graft function. Transplantation. 2010;89(2):194–9. doi:10.1097/TP.0b013e3181c3ca11.

8. Wei S, Li J, Lian Z, Chen Y, Liu Z, You H, et al. Expression of glucocorticoid-induced tumor necrosis factor receptor ligand in rat graft after liver transplantation. Transplant Proc. 2011;43(5):1971–5. doi:10.1016/j.transproceed.2011.03.054.

9. Huang YL, Wang YZ, Chen JB, Wang F, Kang XP, Xia JJ, et al. Prevention of acute and chronic allograft rejection by combinations of tolerogenic dendritic cells. Scand J Immunol. 2011;73(2):91–101. doi:10.1111/j.1365-3083.2010.02485.x.

10. van Kooten C, Lombardi G, Gelderman KA, Sagoo P, Buckland M, Lechler R, et al. Dendritic cells as a tool to induce transplantation tolerance: obstacles and opportunities. Transplantation. 2011;91(1):2–7.

11. Ezzelarab M, Thomson AW. Tolerogenic dendritic cells and their role in transplantation. Semin Immunol. 2011. doi:10.1016/j.smim.2011.06.007.

12. Natarajan S, Thomson AW. Tolerogenic dendritic cells and myeloid-derived suppressor cells: potential for regulation and therapy of liver auto- and alloimmunity. Immunobiology. 2010;215(9–10):698–703. doi:10.1016/j.imbio.2010.05.024.

13. Li J, Meinhardt A, Roehrich ME, Golshayan D, Dudler J, Pagnotta M, et al. Indoleamine 2,3-dioxygenase gene transfer prolongs cardiac allograft survival. Am J Physiol Heart Circ Physiol. 2007;293(6):H3415–23. doi:10.1152/ajpheart.00532.2007.

14. Laurence JM, Wang C, Zheng M, Cunningham S, Earl J, Tay SS, et al. Overexpression of indoleamine dioxygenase in rat liver allografts using a high-efficiency adeno-associated virus vector does not prevent acute rejection. Liver Transpl. 2009;15(2):233–41. doi:10.1002/lt.21662.

15. Medzhitov R, Shevach EM, Trinchieri G, Mellor AL, Munn DH, Gordon S, et al. Highlights of 10 years of immunology in Nature Reviews Immunology. Nat Rev Immunol. 2011;11(10):693–702. doi:10.1038/nri3063.

16. Terness P, Chuang JJ, Bauer T, Jiga L, Opelz G. Regulation of human auto- and alloreactive T cells by indoleamine 2,3-dioxygenase (IDO)-producing dendritic cells: too much ado about IDO? Blood. 2005;105(6):2480–6. doi:10.1182/blood-2004-06-2103.

17. Shumakov VI, Moisiuk Ia G, Shagidulin M. [The evolution of surgical techniques for donor liver procurement]. Vestn Ross Akad Med Nauk. 2006(12):7-11.

18. Cannazza G, Baraldi M, Braghiroli D, Tait A, Parenti C. High-performance liquid chromatographic method for the quantification of anthranilic and 3-hydroxyanthranilic acid in rat brain dialysate. J Pharm Biomed Anal. 2003;32(2):287–93.

19. Banff schema for grading liver allograft rejection: An international consensus document. Hepatology. 1997;25:6.

20. Yu G, Fang M, Gong M, Liu L, Zhong J, Feng W, et al. Steady state dendritic cells with forced IDO expression induce skin allograft tolerance by upregulation of regulatory T cells. Transpl Immunol. 2008;18(3):208–19. doi:10.1016/j.trim.2007.08.006.

21. Ingelsten M, Gustafsson K, Oltean M, Karlsson-Parra A, Olausson M, Haraldsson B, et al. Is indoleamine 2,3-dioxygenase important for graft acceptance in highly sensitized patients after combined auxiliary liver-kidney transplantation? Transplantation. 2009;88(7):911–9. doi:10.1097/TP.0b013e3181b72e49.

22. Terness P, Bauer TM, Rose L, Dufter C, Watzlik A, Simon H, et al. Inhibition of allogeneic T cell proliferation by indoleamine 2,3-dioxygenase-expressing dendritic cells: mediation of suppression by tryptophan metabolites. J Exp Med. 2002;196(4):447–57.

23. Ge W, Jiang J, Arp J, Liu W, Garcia B, Wang H. Regulatory T-cell generation and kidney allograft tolerance induced by mesenchymal stem cells associated with indoleamine 2,3-dioxygenase expression. Transplantation. 2010;90(12):1312–20. doi:10.1097/TP.0b013e3181fed001.

24. Palafox D, Llorente L, Alberu J, Torres-Machorro A, Camorlinga N, Rodriguez C, et al. The role of indoleamine 2,3 dioxygenase in the induction of immune tolerance in organ transplantation. Transplant Rev (Orlando). 2010;24(3):160–5. doi:10.1016/j.trre.2010.04.003.

25. Ghahary A, Li Y, Tredget EE, Kilani RT, Iwashina T, Karami A, et al. Expression of indoleamine 2,3-dioxygenase in dermal fibroblasts functions as a local immunosuppressive factor. J Invest Dermatol. 2004;122(4):953–64. doi:10.1111/j.0022-202X.2004.22409.x.

26. Munn DH, Sharma MD, Lee JR, Jhaver KG, Johnson TS, Keskin DB, et al. Potential regulatory function of human dendritic cells expressing indoleamine 2,3-dioxygenase. Science. 2002;297(5588):1867–70. doi:10.1126/science.1073514.