

Combined therapy of infusion of DC from rats with higher expression of IDO and CD40L on rejection post heart transplantation

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Abstract. – OBJECTIVE: Indoleamine 2, 3-dioxygenase (IDO) can inhibit rejection of graft via inducing T cell apoptosis. CD40L monoclonal antibody (mAb) inhibits T cell activation. However, the effects of the combination of infusion of dendritic cell (DC) from IDO over-expressed donor mice and CD40L mAb on the treatment of graft rejection after heart transplantation have not been reported.

MATERIALS AND METHODS: Allogeneic heart transplantation mouse model was established. Recipient mice were divided into three groups, including control group, IDO group (in which DC donors received adenoviral vector of IDO) and combined therapy group (which received both IDO over-expressed DC infusion and CD40L mAb injection post transplantation). Survival time and cardiac function were observed, with IDO expression being quantified. Flow cytometry (FCM) was used to analyze T cell apoptosis, while enzyme linked immunosorbent assay (ELISA) was adopted to test the levels of interferon- γ (IFN- γ), interleukin-2 (IL-2), interleukin-10 (IL-10) and interleukin-6 (IL-6).

RESULTS: IDO expression was significantly elevated in both IDO and combined therapy groups, with enhanced T cell apoptosis compared to control group ($p < 0.05$). Both groups had better survival time and cardiac functions compared to control group, along with increased IL-10/IL-6 expression and suppressed INF- γ and IL-2 expression ($p < 0.05$). However, combined therapy had a better efficiency compared to IDO group ($p < 0.05$).

CONCLUSIONS: Combined therapy of high IDO expressed mouse DC perfusion with CD40L mAb can elongate the survival time of recipient heart and inhibit rejection reaction via facilitating T cell apoptosis. Meanwhile, combined therapy could also regulate the expression of some immune suppressant factors and mediate the Th1/Th2 cytokine balance.

Key Words:

Heart transplantation, IDO, CD40L, Cytokines, Rejection of transplantation.

Introduction

Insufficient blood supply of heart can be caused by complicated congenital heart diseases, congestive heart failure (CHF), myocardial infarction (MI) or primary myocardial disease (PMD). As a consequence, partial or complete cardiac function can be impaired, leading to the occurrence of cardiac function failure, further blocking blood and nutrient supply for normal tissue/organs, leading to accumulation of toxic metabolites, ion imbalance. Eventually, fatigue, decreased appetite, anemia, or pulmonary edema may occur, or even caused failure of other organs^{1,2}. In modern society, due to working stress, changes of life styles, the incidence of heart failure is rapidly increasing^{3,4}. Currently the best treatment for cardiac dysfunction in terminal stage is heart transplantation⁵. However, graft rejection is observed in some patients, seriously affecting the efficacy of transplantation. In clinical practice, high dosage of immune suppressant reagent is applied to inhibit graft rejection but with unsatisfactory effect sometimes^{6,7}. Therefore, seeking effective managements of graft-induced rejection after heart transplant, without any interruption of body's normal structure/function, or immune system, has become a major focus in the field of heart transplantation⁸. Targeted therapy based on immune pathology or immune modulation drugs are promising approach for the treatment of

rejection in heart-transplanted patients. Serine is a necessary amino acid for human body, and mainly metabolizes via kynurenine pathway. The serine acts by activating the indoleamine 2, 3-dioxygenase (IDO), which is considered as one rate-limiting step for catalyzing pyrrole ring of serine to generate N-methyl-kynurenine, leading to promotion of T cell apoptosis and inhibition of the proliferation of effector T lymphocytes^{9,10}. As the most potent antigen-presenting cell (APC), dendritic cell (DC) plays a critical role in the regulation of immunity after transplantation as the sole APC that can activate primary T cells^{11,12}. Interaction between co-stimulating factors CD40L and CD40 is important for T lymphocytes activation. Therefore, the blocking of such interaction using CD40L monoclonal antibody (mAb) is reported to be able to suppress post-transplant rejection^{13,14}. No study, however, has been performed to investigate the efficiency of combined therapy of CD40L mAb with IDO transfection into donor DC in the prevention of graft rejection after heart transplantation.

Materials and Methods

Experimental Animals

A total of 24 healthy in-breed male C57BL/6(H-2b) mice (provided by Jilin University) were used as the donors, whilst 24 healthy in-breed Balb/C(H-2d) mice were used as the recipients. All animals aged between 6 to 8 weeks, with body weight 22 ± 4 g. Animals were purchased and kept in an specific-pathogen-free (SPF) grade facility with fixed temperature ($21 \pm 1^\circ\text{C}$) and relative humidity (50-70%) under 12 h/12 h light cycle. Mice were used for all experiments, and all procedures were approved by the Animal Ethics Committee of China-Japan Union Hospital of Jilin University (Changchun, China).

Reagents and Equipment

All surgical instruments were purchased from Suzhou Instrument (Suzhou, China). Surgical microscope was purchased from Olympus (Mode: CX23, Tokyo, Japan). Roswell Park Memorial Institute-1640 (RPMI-1640) medium was purchased from Gibco (Grand Island, NY, USA). Recombinant mouse colony stimulating factor (rmGM-CSF), recombinant mouse tumor necrosis factor α (rmTNF- α) and interleukin-4 (rmIL-4) were purchased from Peprotech. Co. Ltd. (Rocky Hill,

NJ, USA). CD40L monoclonal antibody (mAb) was purchased from BD Biosciences (Franklin Lakes, NJ, USA). Adenoviral vector (AdV) for IDO was purchased from GeneReach Biotechnology Corp. (Xiamen, China). HEK293 cell was purchased from American Type Culture Collection (ATCC Cell Bank, Manassas, VA, USA). Polyvinylidene difluoride (PVDF) membrane was purchased from Pall Life Sciences (Covina, CA, USA). Western blot chemical reagents were purchased from Beyotime Biotech. (Shanghai, China). Enhanced chemiluminescence (ECL) reagent was obtained from Amersham Biosciences (Piscataway, NJ, USA). Rabbit anti-mouse IDO mAb, and goat anti-rabbit horseradish peroxidase (HRP) conjugated IgG secondary were purchased from Cell Signaling Technology (Danvers, MA, USA). RNA extraction kit and reverse transcription kit were purchased from ABI (Foster City, CA, USA). Enzyme linked immunosorbent assay (ELISA) kits for interferon γ (INF- γ), IL-2, IL-10 and IL-6 were purchased from R&D systems Inc. (Minneapolis, MN, USA). Lab system microplate reader was purchased from Bio-Rad Laboratories (Version: 1.3.1, Hercules, CA, USA). DNA amplification cyclor was purchased from PE Gene Applied Biosystems. (Mode: 2400, Foster City, CA, USA). ABI 7500 Real-Time PCR cyclor was purchased from ABI (Foster City, CA, USA). FACS Aria II flow cytometry equipment was purchased from BD Biosciences (Franklin Lakes, NJ, USA). Other common reagents were purchased from Sangon Biotech. Co. Ltd. (Shanghai, China).

Amplification of AdV-IDO Recombinant Viral Vector

A total of 4×10^5 /ml HEK293 cells were digested and cultured in 6 cm culture dish for 24 h until 80% confluence. The supernatant was discarded with inoculation of 100 μl viral suspension. The mixture was gently mixed and was cultured in a chamber for 72 h. Transfected cells along with supernatants were collected through centrifuging at $10000 \times g$ for 20 min at 4°C . After filtration, cells and supernatants were collected and frozen at -80°C .

DC Preparation and AdV-IDO Transfection

Under sterile conditions, bilateral femur and tibia were collected from C57BL/6 mice. Phosphate buffer saline (PBS) was used to rinse bone marrow cavity for collecting bone marrow cells.

After rinsing in culture medium, bone marrow cells were re-suspended in complete RPMI-1640 culture medium, and inoculated into 6-well plate at 2×10^6 /ml density. Cells were cultured in a humidified chamber at 37°C with 5% CO₂ for 2 h. The supernatant was removed to obtain attached cells. Fresh medium containing 1000 IU/ml rmGM-CSF, 500 U/ml rmIL-4 and 500 U/ml rmTNF- α was added for 7-day incubation, with medium being changed every 2-3 days. Suspended cells were collected as DC. AdV-IDO with multiplicity of infection (MOI) = 200 was transfected into 2×10^5 /ml DC. 2 h after transfection, viral medium was discarded. Cells were then re-suspended in complete RPMI-1640 medium for 2 h continuous culture.

Animal Grouping

Recipient mice was randomly assigned into three groups (n=8 each). In control group, recipient mice did not receive any treatment before surgery. In IDO group, 1×10^6 AdV-IDO transfected DCs were infused via tail veins 5 days and 3 days before heart transplantation. In combined treatment group, after pre-op transfusion of DCs over-expressing IDO, 250 μ g/d CD40L mAb were applied at 0 d, 1 d, 2 d and 4 d after transplantation.

Establishment of Mouse Heterotopic Heart Transplantation Model

Donor mice were fasted 12 h before surgery, anesthetized by 10% hydrate chloral and fixed on the table. Heart was exposed via layered incisions in middle chest. Synchronized anesthesia was performed. After fixation on a supine position, aorta was ligated at distal site and was clapped at proximal site to remove donor heart. Inferior vena cava and abdominal aorta of recipient mice were connected to pulmonary artery and ascending aorta of donor heart, which was then transplanted into the abdominal site. The successful transplantation was examined by daily check of pulsation of transplanted heart. Those with continuous pulsation over 3 days were identified as successful transplantation.

Sample Collection

Seven days after transplantation, blood samples were collected from abdominal aorta by negative pressure tubes. After 30 min room temperature incubation, blood samples were centrifuged at 4°C for 10 min under 3600 r/min. The supernatant was saved and frozen at -20°C for further use. Heart tissues were cut and kept at -80°C for further use.

Survival and Physiological Indexes of all Donor Mice

Survival times and physiological status including food intake, mental status and motility, were monitored after transplantation.

Measurement of Levels of Lactic Dehydrogenase (LDH) and Creatine Kinase (CK)

Blood was draw from mice on day 7 after transplantation followed by serum isolation for analysis of the levels of Lactate Dehydrogenase (LDH) Assay Kit (Colorimetric, Abcam Biotech., Cambridge, MA, USA) and Creatine Kinase (CK) Activity Assay Kit (Colorimetric, Abcam Biotech., Cambridge, MA, USA) by commercial kits according to manufacturer's instructions.

Real-Time PCR for IDO mRNA Expression

TRIzol reagent was used to extract RNA from all heart tissues. DNA reverse transcription was performed following the manual instruction. Premier 6.0 was used to design all primer sequences, which were synthesized by Invitrogen/Life Technologies (Carlsbad, CA, USA) as shown in Table I. Real-time PCR was performed under the following conditions: 55°C for 1 min, followed by 92°C for 30 s, 58°C for 45 s and 72°C for 35 s, in 35 cycles. Data were collected and analyzed by built-in software of PCR cycler. Using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the internal reference, fluorescent quantification was performed to calculate CT values of all samples and standards. Using cycle threshold (CT) values of standards as the reference, a standard curve was plotted for semi-quantitative analysis by $2^{-\Delta Ct}$ method.

Table I. Primer sequence.

| Gene | Forward primer 5'-3' | Reverse primer 5'-3' |
|-------|----------------------|-----------------------|
| GAPDH | ACCAGGTATCTGCTGGTTG | TAACCATGATGTCAGCGTGGT |
| IDO | GGTCATTGACCTATGACTG | TAACTTCCGCTTCGTCCTTA |

Western Blot Analysis of IDO Protein Expression

Total proteins were extracted from heart tissues. In brief, liquid nitrogen was used to homogenize tissues, which were mixed with lysis buffer for iced incubation for 15-30 min. Cells were ruptured under ultrasound (5 s, 4 times) followed by 10000 ×g centrifugation (4°C, 15 min). The supernatant was saved, quantified for protein levels and kept at -20°C for Western blot assays. Proteins were separated in 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto polyvinylidene difluoride (PVDF) membrane using semi-dry method (160 mA, 1.5 h). Non-specific binding was blocked by 5% defatted milk powder for 2 h. IDO monoclonal antibody (1:1000 dilution) was added for overnight incubation at 4°C. After PBS Tween-20 (PBST) washing, 1:2000 diluted goat anti-rabbit secondary antibody was added for dark incubation for 30 min. With PBST washing, chromogenic substrate was added for 1 min development. With X-ray exposure, band density was measured for calculating relative expression. Each group was tested for four times (n=4) for further analysis.

ELISA Analysis of Serum Cytokine Secretion

Mouse serum was extracted to test serum cytokine levels including IFN- γ , IL-2, IL-10 and IL-6 following manual instruction of ELISA kit. Optical density (OD) values of standard samples were calculated for plotting linear regression function, on which sample concentration was deduced based on their OD values.

Flow Cytometry Measurement of T Cell Apoptosis

7 days after surgery, peripheral blood samples were collected for cell lysis. After washing in cold PBS, anti-CD3 monoclonal antibody with APC labeling was added for 2 min incubation. The supernatant was discarded after centrifugation. A total of 5 μ l Annexin-V with PI were added for 10 min incubation, followed by addition of 400 μ l binding buffer. The sample was loaded for test on flow cytometry.

Statistical Analysis

SPSS19.0 software (IBM Corp., IBM SPSS Statistics for Windows, Armonk, NY, USA) was used for data analysis. Measurement data were presented as mean \pm standard deviation (SD).

Tukey's post-hoc test was used to validate the ANOVA for comparing measurement data between groups. LSD test was employed to compare between groups. A statistical significance was defined when $p < 0.05$.

Results

General Conditions of Mice

Mice survival time and average survival periods were observed in all groups. Results showed good mental status, normal food intake and motility, with late onset of rejection as well as significantly elongated survival time after IDO or combined treatment ($p < 0.05$ compared with control group). In combined treatment group, mice can even live as long as 23 days (Figure 1).

Myocardial Function Analysis

Seven days after surgery, CK and LDH levels were analyzed in all groups. In both IDO and combined treatment group, CK and LDH levels were significantly decreased ($p < 0.05$ compared with control group). However, the levels of CK and LDH were improved in combined therapy group ($p < 0.05$, Figure 2).

IDO mRNA Expression in Heart Tissues

Real-time PCR was used to test expression level of IDO mRNA in heart tissues from all groups. Results showed significantly elevated IDO mRNA expression in both IDO and com-

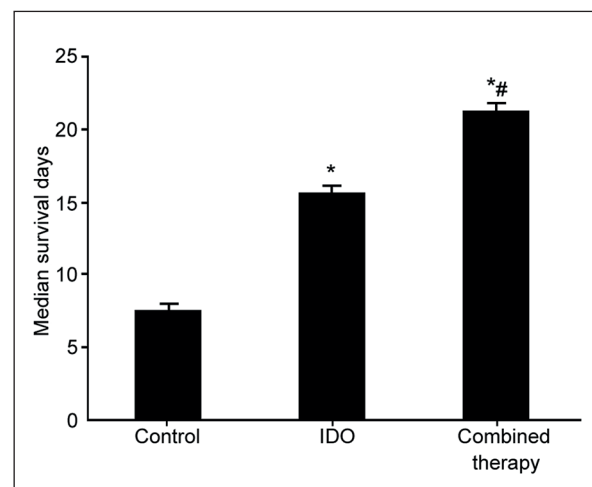


Figure 1. Survival days of all groups after heart transplantation. * $p < 0.05$ compared to control group. # $p < 0.05$ compared to IDO group.

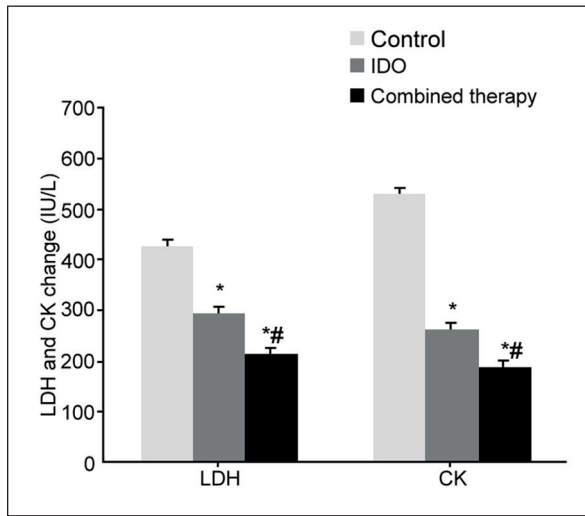


Figure 2. Cardiac function indexes of mice. * $p < 0.05$ compared to control group. # $p < 0.05$ compared to IDO group.

combined therapy mice after AdV-PD-IDO transfection ($p < 0.05$ compared to control group). However, no significant difference was found between IDO and combined therapy group ($p > 0.05$, Figure 3).

IDO Protein Expression Profile in Cardiac Tissues

Western blot was employed to test IDO protein expression in all heart tissues. Results showed similar results as those of IDO mRNA. In both IDO and combined therapy mice, after AdV-PD-IDO treatment, IDO protein expression was significantly elevated in heart tissues

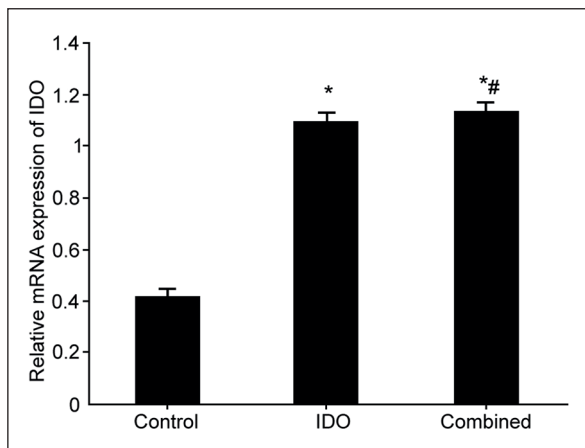


Figure 3. IDO mRNA expression in all heart tissues. * $p < 0.05$ compared to control group.

($p < 0.05$ compared to control group). No statistically significant difference existed between IDO and combined therapy group ($p > 0.05$, Figure 4).

T Cell Apoptosis in Mice

After IDO or combined therapy, apoptotic rate of peripheral blood CD3+ T lymphocytes was $8.47\% \pm 1.21\%$ in control group, and was $16.81\% \pm 2.75\%$ in IDO treated mice and was $41.21\% \pm 5.12\%$ in combined therapy group. Both IDO and combined therapy significantly increased the apoptotic rate of peripheral CD3+ T lymphocytes ($p < 0.05$ compared to control group). However, combined therapy achieved more significant apoptosis of peripheral CD3+ T cells ($p < 0.05$ compared to IDO group, Figure 5).

Changes of Serum Cytokines for All Mice

ELISA was used to test the changes of cytokines in mouse serum. Results showed significantly decreased serum INF- γ or IL-2 expression in either of IDO or combined therapy group ($p < 0.05$ compared to control group). In combined therapy group, the decrease of serum cytokine levels was even more significantly ($p < 0.05$ compared to IDO group, Figure 6).

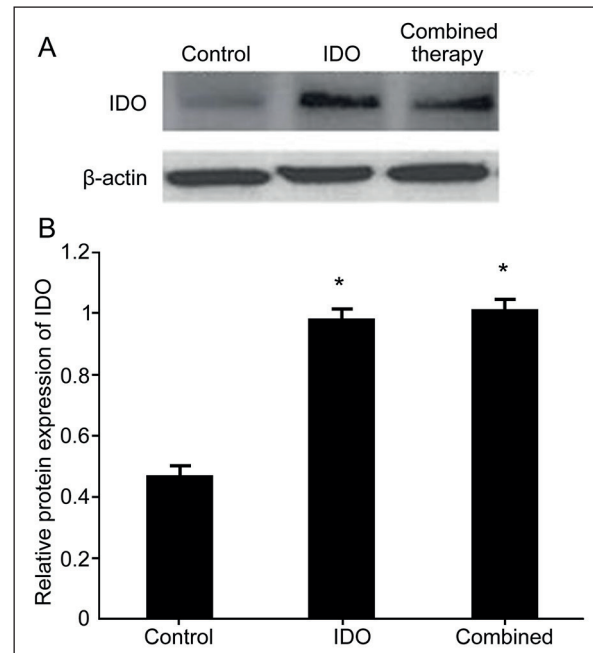


Figure 4. IDO protein expression in all cardiac tissues. * $p < 0.05$ compared to control group.

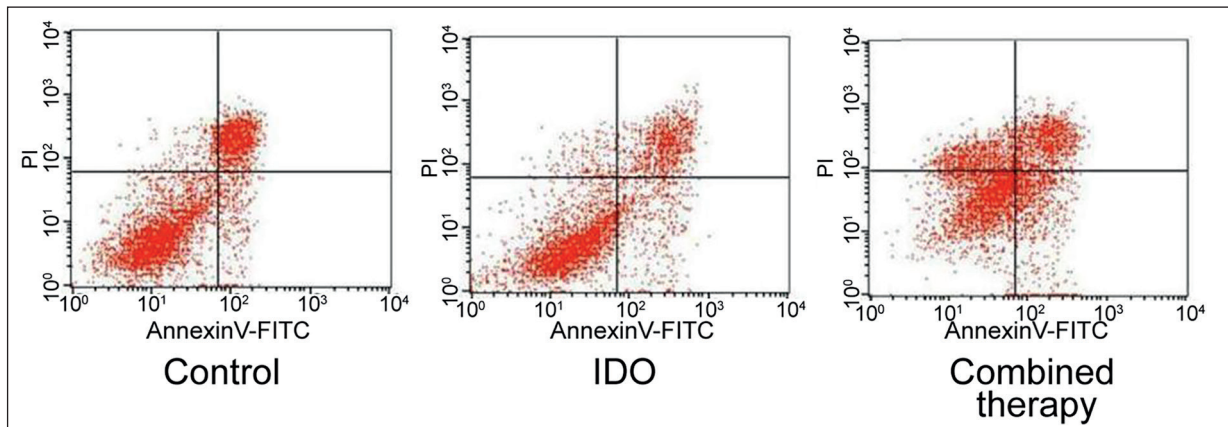


Figure 5. Analysis of T cell apoptosis.

Discussion

The determining factor of survival time for heart transplant patients is to overcome rejection response, therefore improving life quality of patients. Currently, transplanted patients mainly rely on immunosuppressant to overcome such rejection response to increase survival rate. Consequent adverse reactions such as infection or tumor occurrence, chronic rejection response have also revealed¹⁵. IDO, as one mono-enzyme of hemoglobin, is the rate-limiting enzyme in

serine-kynurenine metabolism pathway. Current study showed that IDO can lead to exhausted serine in T lymphocytes, producing catabolic products to cause T cell apoptosis, and possibly leading to regulatory T cell proliferation^{16,17}. IDO exerts important roles in maternal-fetal immune tolerance, auto-immune disease, tumor escape and transplant immune tolerance. Adenoviral vector has been widely applied in gene therapy due to high efficiency, safety, broader host range and high viral titer¹⁸. IDO is mostly expressed on macrophage or DC, which can elevate IDO

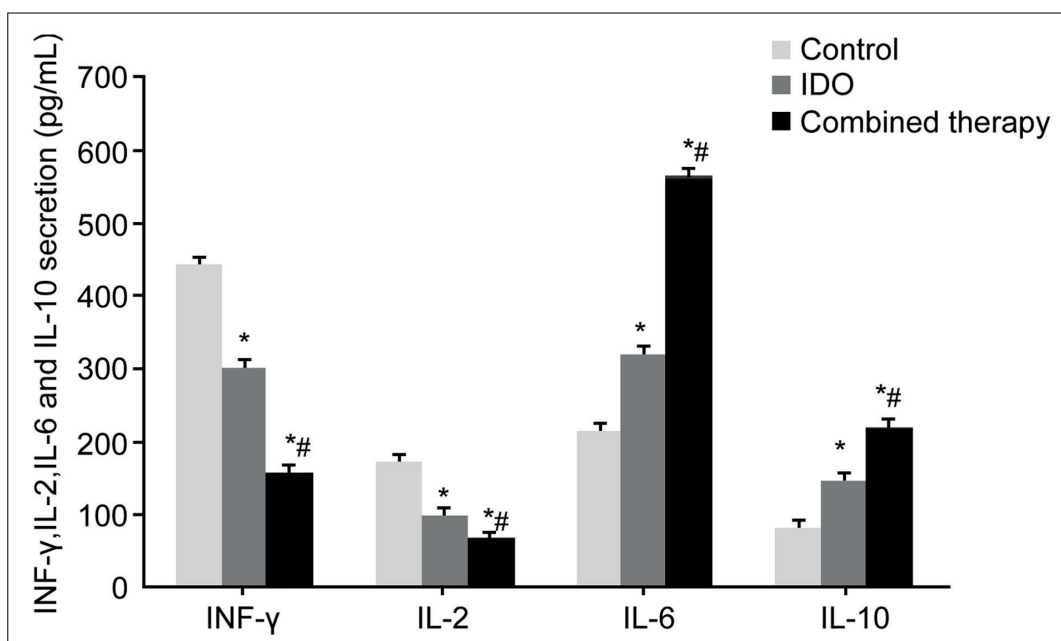


Figure 6. Expressional change of serum cytokine factors on all mice. * $p < 0.05$ compared to control group. # $p < 0.05$ compared to IDO group.

expression¹⁹. Therefore this study firstly utilized adenovirus to induce IDO over-expression and further transfection into DC. Results showed that adenovirus induced IDO gene transfection into DC, once being infused into recipient mice, can facilitate IDO gene/protein expression in transfected mice and significantly elongated heart survival time and better life quality compared with control group. These results suggest that IDO over-expression effectively improved heart functions. Other studies showed that CD40L worked as one necessary co-stimulus molecule for T cell activation. Via binding to CD40, CD40/CD40L can interact with downstream tyrosine kinase signal molecules to potentiate T cell proliferation, and modulate primary immune response, as well as the functions of memory/effector T cells. These results suggest that its critical roles in mediating immune rejection response^{20,21}. This study further explored the effects of transfusion of IDO high-expressed DC and mAb of CD40L on the rejection response of mouse heart. This result demonstrated that such combined therapy had significantly better inhibitory effects on rejection of heart transplantation compared to those in IDO group, as shown by elongated heart survival time and improved CK/LDH levels. This result also suggests that that combined therapy had more significantly inhibitory effects on rejection response. Following mechanistic study found that the combined therapy using transfusion of highly-expressed IDO of DC and CD40L mAb could facilitate T cell apoptosis and inhibit T cell proliferation, possibly associated with proliferation inhibition or apoptosis potentiation as a result of insufficient protein synthesis after antigen presenting by DC, leading to inhibition of T cell mediated immune response²². Helper T lymphocytes and their cytokines have become critical factors during acute immune rejection²³. Under antigen stimuli, T cell sub-population is further differentiated into Th1, which secretes IFN- γ and IL-2, and Th2 for secreting IL-6 and IL-10. During occurrence of acute rejection response, large amounts of Th1 cytokines were produced by transplanted organs, whilst Th2 cytokines dominate in transplanted organs to inhibit immune response or induce immune tolerance via suppressing lymphocyte maturation and differentiation^{24,25}. This study found that combined therapy could inhibit synthesis and secretion of Th1 cytokines, thus impeding transplant-associated rejection, consistent with immune tolerance after transplantation²⁴⁻²⁶.

Conclusions

We found that over-expression of IDO in donor mice DC for transfusion, plus CD40L mAb, could prolong recipient heart survival time and inhibit transplantation rejection via facilitating T cell apoptosis, inhibiting the expressions of immune suppression factors, as well as regulating Th1/Th2 cytokine homeostasis.

Acknowledgements

This project was supported by the Liaoning Natural Science Foundation (No. 2013021011).

Conflict of Interest

The Authors declare that they have no conflict of interests.

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