

Original Article

Effects of Foxp3 gene modified dendritic cells on mouse corneal allograft rejection

Yu-Bo Gong^{1,2}, Lian-Na Hu¹, Yong Liu³, Gen-Cheng Han⁴, Hui-Ling Guo¹, Ling Luo¹, Li-Qiang Wang², Yan Li⁴, Yi-Fei Huang²

¹Department of Ophthalmology, Chinese People's Liberation Army (PLA) 306th Hospital, Beijing 100101, China;

²Department of Ophthalmology, Chinese People's Liberation Army (PLA) General Hospital, Beijing 100853, China;

³Department of Ophthalmology, General Hospital of Air Force, Beijing 100142, China; ⁴Department of Molecular Immunology, Institute of Basic Medical Sciences, Academy of Military Medical Sciences, Beijing 100850, China

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Abstract: Objective: To investigate the effect of Foxp3 gene modified dendritic cells (Foxp3 + DC) on allogeneic T cells proliferation and to study the effect of Foxp3 + DC on corneal allograft rejection. Methods: Lentivirus-Foxp3 was transfected into DC2.4 cells, as Foxp3 + DC cells. 42 BALB/c mice were randomly divided into: Group A (n = 6), normal group; Group B (n = 12), Group C (n = 12) and Group D (n = 12), allograft groups, were treated with normal saline, DC2.4, Foxp3 + DC by intraperitoneal injection, respectively. Results: Compared with the control group, Foxp3 protein in the Foxp3 + DC cells increased significantly ($P < 0.05$); the expressions of CD80 and CD86 immunophenotypes of Foxp3 + DC cells decreased significantly ($P < 0.05$); IL-12 secretion reduced ($P < 0.05$), but IL-10 secretion was promoted ($P < 0.05$). The average transplant survival time in Group B was (14.833 ± 1.472) d, and Group C and Group D led to a statistically significant prolongation of transplant survival to (17.667 ± 1.366 , 23.000 ± 2.000) d ($P < 0.05$) respectively. 14 d after transplantation, as compared with Group C and D, the expressions of IFN- γ in grafts markedly increased in Group B. 14 d after transplantation, as compared with Group B, the expressions of Foxp3 mRNA, IDO mRNA in grafts decreased remarkably in Group C and D ($P < 0.05$); as compared with Group C, the expressions of Foxp3 mRNA, IDO mRNA in grafts decreased remarkably in Group D ($P < 0.05$). Conclusion: Foxp3 + DC cells reduce the expression of costimulatory factors, reduce the secretion of IL-12, promote IL-10 production and inhibit the stimulation of alloreactive T cell proliferation response capacity. Foxp3 + DC cells play important roles in inhibiting corneal allograft immune response and prolonging graft survival time.

Keywords: Foxp3, lentivirus, DC2.4, corneal transplantation, immune tolerance

Introduction

Immunological rejection of corneal allograft is a main cause of transplant failure. Therefore it is of great significance for the normal corneal transplantation, especially high-risk corneal transplantation that how to avoid or suppress corneal allograft rejection and prolong graft survival time of corneal transplantation.

Dendritic cells (DCs) are not only involved in immune response against foreign antigens but also play an important role in inducing immune tolerance. Studies have shown that DC-induced immune tolerance is mainly achieved through inducing T cell anergy and T cell clone deletion and promoting immunosuppression of regulatory T cells [1, 2]. So how to induce the formation of tolergenic DC has become a hot research

field of transplantation immunology. With the development of genetic engineering, genetic modification of dendritic cells has become an important way to induce transplantation tolerance. DC by transgenic technology which allows high expressions of TGF- β , CTLA-4, IDO, FasL can prolong the survival of transplanted organs by inhibiting the T cells function [3-5]. Foxp3 which is an important negative regulator protein, keeps the CD4 + CD25 + regulatory T cells (CD4 + CD25 + Tr) development and maintenance of immune function. Foxp3 plays pivotal roles in regulating the development and functioning of CD4 + CD25 + Tr [6-8]. Recent studies have shown that, Foxp3 plays a negative regulatory role in DCs; DCs transfected with adenovirus-mediated Foxp3 gene result in significantly inhibiting T cell proliferation and Th1-type immune response [9].

Foxp3 gene modified dendritic cells affect mouse corneal allograft rejection

The present study aimed to investigate the effects of Foxp3 gene modified dendritic cells (Foxp3 + DC) immune suppression in vitro and to further explore the preventive effects of Foxp3 + DC on the corneal allograft rejection.

Materials and methods

Animals

C57BL/6 mice (male; specific pathogen free), aged 6-8 weeks old, weighing 18 ~ 22 g and BALB/c mice (male; specific pathogen free), aged 6-8 weeks old, weighing 18 ~ 22 g were purchased from the Experimental Animal Center of the Chinese PLA General Hospital. All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals as adopted by the Experimental Animal Center, Chinese PLA General Hospital. This study was approved by the ethics committee of the Chinese PLA General Hospital.

Reagent

Lentiviral vector system was purchased from Genechem Company, the virus packaging system consists of pGC-FU carrier, pHelper 1.0 carrier and pHelper 2.0 carrier. Mouse Foxp3 cDNA and DC2.4 cell line was provided by the Department of Molecular Immunology of Institute of Basic Medical Research at Academy of Military Medical Sciences. E. coli DH5 α (TaKaRa), Lipofectamine 2000 transfection reagent and Opti-MEM (Invitrogen), plasmid extraction kit (Qiagen), In-Fusion™ PCR Cloning Kit (Clontech), TRizol (Invitrogen), anti-transcription kit (TransGen), PE-labeled anti-mouse Foxp3 mAb and matched isotype control (eBioscience), Other reagents used included fixation/permeabilization solution (eBioscience), 10 × permeabilization buffer (eBioscience), PE-labeled anti-mouse CD80 mAb, CD86 mAb, and matched isotype control (BD), mouse IL-12, IL-10 ELISA kit (Dakewe), CCK-8 (Dojindo), RPMI-1640 culture medium (Hyclone), rabbit anti-mouse IFN- γ (Bioss); rabbit Streptavidin-HRP kit (Cwbio).

Construction, identification and packaging of recombinant lentivirus targeting Foxp3

Preparation of target gene by PCR

The design and synthesis of primers for mouse Foxp3 were carried out in GeneChem Co., Ltd

as follows: Foxp3-Age I-F: 5'-GAGGATCCCCGGG TACCGTCCGACCATGCCCAACCCTAGG-CCAG-3' and Foxp3-Age I-R: 5'-TCACCATGGTGGCGACCGGAGGGCAGGGATTGG-AGCAC-3'. The I-F primer contains exchange of base pairs, Age I restriction site (underline), expressed sequence tag (double underline) and 5' sequence for synthesis of target gene. The I-R primer contains exchange of base pairs, Age I restriction site (underline), and 3' sequence for synthesis of the target gene. The anticipated size of products is 1333 bp. The products were subjected to gel electrophoresis and the target gene was identified.

Construction of the lentivirus vector carrying pGC-FU-Foxp3

PCR products were inserted into lentiviral vectors through exchange. In brief, purified PCR products were directionally introduced into the DNA of pGC-FU lentiviral vectors with in-fusion base-exchange enzyme (digestion of pGC-FU lentiviral vectors with restriction enzyme Age I). Then, the lentivirus was used to transform competent cells (DH5 α) and positive colonies were selected for identification and sequencing (Invitrogen). PCR was performed for the identification of primers (anticipated size was 1494 bp), Ubi-F: 5'-GGGTCAATATGTAATTTTCAGTG-3' (this primer was in the promoter of Ubiquitin), EGFP-N-R: 5'-CGTCGCCGTCAGCTCGACCAG-3' (this primer was in the N-terminus of EGFP).

Packaging of Lentivirus-Foxp3

Different plasmids (pGC-FU-Foxp3, pHelper 1.0 and pHelper 2.0) were mixed with Opti-MEM of equal volume, which was then added to Lipofectamine 2000 solution. This mixture was subsequently added to 293T cells followed by incubation at 37°C in an atmosphere with 5% CO₂ for 48 h. After transfection, the supernatant was collected followed by centrifugation and filtration enrichment. Aliquots of lentivirus solution were stored at -70°C for use.

Transfection of DC2.4 cells with Lentivirus-Foxp3

DC2.4 cells were seeded in 96-well plate at a density of 5 × 10³ cells/ml at 1 day before transfection. Lentivirus solution (MOI = 20) was added to cells followed by complete blending. Then, the cells were incubated for 8 h and the cells were observed. Then, the medium was

Foxp3 gene modified dendritic cells affect mouse corneal allograft rejection

refreshed and fluorescent protein was observed under fluorescence microscope.

Flow cytometry for Foxp3 after transfection with Lentivirus-Foxp3

Four days after transfection, DC2.4 cells in the control group, the Lentivirus group and the Lentivirus-Foxp3 group were collected and washed with PBS twice. These cells were re-suspended in PBS at a density of $1 \sim 2 \times 10^6$ /mL. Adding cell suspension in each tube 200 μ L, 2 000 rpm, centrifuged 5 min, and then the cell pellets were treated with 400 μ L of permeabilization buffer (1:3 dilution). The cells were placed in dark overnight. After centrifugation, the cell pellets were washed once with PBS and once with $1 \times$ permeabilization buffer, followed by addition of $1 \times$ permeabilization buffer to resuspend the cells to a final volume of 200 μ L. 1 μ L of PE-labeled anti-mouse Foxp3 mAb or an equal amount of isotype control was added to the cells. The cells were then kept in dark at 4°C for 30 min before centrifugation at 1000 r/min for 10 min. The resultant cell pellets were washed with $1 \times$ permeabilization buffer and subsequently fixed in 200 μ L of 1% paraformaldehyde solution. The cells were kept at 4°C in dark until analysis.

Flow cytometry for CD80, CD86 expressions after transfection with Lentivirus-Foxp3

Four days after transfection, DC2.4 cells in the control group, the Lentivirus group and the Lentivirus-Foxp3 group were collected and washed with PBS twice. These cells were re-suspended in PBS at a density of $1 \sim 2 \times 10^6$ /mL. Cell suspension (200 μ L) and PE conjugated anti-mouse CD80 mAb (1 μ L) or CD86 mAb (1 μ L) were added to tubes followed by incubation at 4°C for 30 min in dark. Then, centrifugation was performed at 2000 r/min for 5 min, and supernatant was removed. Cells were washed with PBS twice and fixed in 200 μ L of 1% paraformaldehyde solution. The cells were kept at 4°C in dark until analysis.

ELISA for IL-12, IL-10 levels in the culture supernatant

Four days after transfection, Cell culture supernatants in the control group, the Lentivirus group and the Lentivirus-Foxp3 group were collected and centrifuged at 5000 r/min for 5 min. The levels of IL-12, IL-10 in the supernatants

were determined using a mouse IL-12, IL-10 ELISA kit following the manufacturer's instructions.

Mixed lymphocyte reaction

Four days after transfection, DC2.4 cells in the control group, the Lentivirus group and the Lentivirus-Foxp3 group were collected and washed with PBS twice. Each group cell suspensions (1×10^6 /mL) were taken and mitomycin C was added to a final concentration of 30 μ g/mL. The mixture was incubated in water wash at 37°C for 30 min before centrifugation at 2000 r/min for 5 min. The cell pellets were washed twice with RPMI-1640 medium and centrifuged at 2000 r/min for 5 min after each wash. The cell pellets were resuspended in the RPMI-1640 medium to a final concentration of 1×10^6 /mL and these cells were used as stimulator cells. Meanwhile, single cell suspension was prepared by harvesting the spleen of BALB/c mice under sterile conditions; lymphocytes were isolated by use of lymphocyte separation medium and the cell density was adjusted to 1×10^6 /mL. These cells were used as responding cells. 100 μ L of stimulator cells or responding cells were added to each well on the 96-well plates and the responding cells were used as controls. Each sample was tested in triplicates. The cells were incubated in an incubator (37°C, 5% CO₂) for three days. 20 μ L of CCK-8 was added to each well 4 h before the end of culture. After culture, the optical absorbance values at 450 nm were recorded by a microplate reader. The stimulation index (SI) was calculated as optical absorbance values of experimental group at 450 nm/optical absorbance values of control group at 450 nm. The results reported were the mean of three wells.

Grouping

A total of 18 C57BL/6 mice were used as donors and 42 BALB/c mice as recipients. These BALB/c mice were randomly divided into 4 groups. Mice in Group A (n = 6) did not receive treatment and those in the other 3 groups received corneal transplantation. Mice in Group B (n = 12) were treated with normal saline in which 6 mice were used for observation of corneal rejection and survival time. Mice in the Group C (n = 12) were treated with DC2.4 3×10^6 cells in which 6 mice were used for observation of corneal rejection and survival time. Mice in the Group D (n = 12) were treated with

Foxp3 gene modified dendritic cells affect mouse corneal allograft rejection

Foxp3 + DC 3×10^6 cells in which 6 mice were used for observation of corneal rejection and survival time. Intraperitoneal injection was performed on preoperative third day, respectively.

Modeling

The right eyes of recipient mice underwent transplantation and the donor mice provided bilateral corneas. Tropicamide drops and tetracaine 1% drops were administered thrice 15 min before surgery. A 1.5 mm diameter full thickness corneal disc was trephined from the center of the donor cornea and grafted into a 1.5 mm graft bed of the recipient cornea, which was then secured with 6-8 interrupted 11/0 nylon sutures. Sutures were cut as short as possible, but were not removed. Aseptic technique was used throughout the surgery and sterilized air was injected forming anterior chamber. The 7th day after surgery, corneal sutures were removed.

Observation of mice undergoing allograft corneal transplantation

Rejection reaction was observed daily with a slitlamp microscope. The criteria for rejection were previously reported by Sonoda Y and Streilein JW [10]. Scores of corneal opacity: 0, completely transparent cornea; 1, mild epithelial opacity in the cornea; 2, anterior stromal opacity and deteriorated opacity in cornea with observable iris; 3, mild opacity in the deep matrix with blur iris; 4, moderate opacity in the deep matrix with observable pupil only; 5, complete opacity in the cornea with invisible anterior chamber. Scores of neovascularization: 0, no vessels; 1, newly generated vessels around the recipient bed; 2, newly generated vessels around the graft; 3, newly generated vessels in a majority of graft; 4, newly generated vessels in all over the graft. The corneal graft rejection was determined by the scores of corneal opacity and neovascularization. One week after transplantation, the suture was removed. The score of cornea opacity of ≤ 2 suggests improvement; the score of ≥ 3 indicates deteriorated opacity; the score of cornea opacity of ≥ 3 and that of neovascularization of ≥ 2 imply rejection. The time to rejection was recorded, which was the survival time of graft, and the survival time of grafts in different groups was compared. Infection, hyphema, disappearance

of anterior chamber, and severe edema, opacity and destruction of cornea within 5 days after transplantation were not defined as signs of rejection. Animals were added if necessary.

Pathological examination and immunohistochemistry

The cornea graft was obtained 14 days after transplantation. Animals were anesthetized and the cornea graft was collected. Grafts were fixed in 10% formaldehyde and embedded in paraffin followed by pathological examination. In addition, immunohistochemistry for IFN- γ was also carried out according to manufacturer's instructions. Counterstaining was performed with hematoxylin.

Detection of mRNA expressions of Foxp3 and IDO in the cornea graft

The corneas of different groups (n = 4 per group) were obtained at 14 days after transplantation and total RNA was extracted. The corneas were grinded in liquid nitrogen and then transferred into 1 ml of TRIzol followed by extraction of total RNA according to manufacturer's instructions. Then, the RNA was subjected to reverse transcription into cDNA and $2^{-\Delta\Delta Ct}$ method was employed to calculate the relative expression of target gene. The primers were as follows: for Foxp3: 5'-CCCAGGAAAGACAGC-AACCTT-3' (forward), 5'-TTCTACAACCAGGCCACTTG-3' (reverse); for IDO: 5'-GTCCGTGAGTTTGTGTCATT-3' (forward), 5'-GTATCTACTATTGCGAGGTG-3' (reverse); for GAPDH, 5'-TCTTGGGCT-ACACTGAGGAC-3' (forward), 5'-CATACCAGGAAATGAGCTTGA-3' (reverse). The conditions for PCR included a total of 40 cycles of 95°C for 10 min, 95°C for 20 s, 60°C for 15 s and 72°C for 10 s. The melt curve was delineated at 60°C ~ 95°C.

Statistical analysis

The quantitative data were presented as mean \pm standard deviation ($\bar{X} \pm SD$) and statistical analysis was performed with statistic software SPSS 11.5. One Way ANOVA was used for comparisons between multiple groups. Multiple comparisons between means were performed with LSD method. A value of $P < 0.05$ was considered statistically significant.

Foxp3 gene modified dendritic cells affect mouse corneal allograft rejection

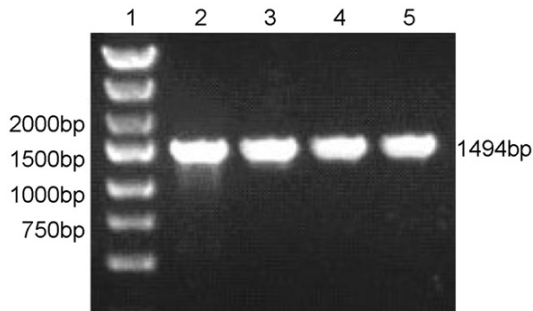


Figure 1. Identification by PCR of the constructed vector (lane 1: DNA Marker; lane 2-5: transforming positive clones).

Results

Identification of the lentivirus vector pGC-FU-Foxp3 and sequencing

Positive colonies were selected followed by PCR amplification. Results showed the size of products from lentivirus vectors was identical to that of amplified products (1 494 bp). Sequencing revealed the length of inserted gene was 1290 bp (mouse Foxp3 gene). These findings suggested the construction of lentivirus vectors pGC-FU-Foxp3 was successful (**Figure 1**).

Expressions of Foxp3 in Lentivirus-Foxp3 infection DC2.4 cells

The percentage of Lentivirus-Foxp3 infection in DC2.4 cells that expressed Foxp3 was (1.59 ± 0.14)% in the control group, (1.52 ± 0.16)% in the Lentivirus infection group, and (54.18 ± 4.59)% in the Lentivirus-Foxp3 infection group. Compared with the control group and Lentivirus group, the Foxp3 was significantly higher in Lentivirus-Foxp3 group ($P < 0.05$).

Expressions of CD80, CD86 in Lentivirus-Foxp3 infection DC2.4 cells

The percentage of Lentivirus-Foxp3 infection in DC2.4 cells that expressed CD80 was (92.34 ± 2.70)% in the control group, (90.89 ± 2.48)% in the Lentivirus infection group, and (57.76 ± 1.69)% in the Lentivirus-Foxp3 infection group. Compared with the control group and Lentivirus group, the CD80 was significantly lower in Lentivirus-Foxp3 group ($P < 0.05$).

The percentage of Lentivirus-Foxp3 infection DC2.4 cells that expressed CD86 was (97.20 ±

1.47)% in the control group, (97.07 ± 1.62)% in the Lentivirus infection group, and (64.40 ± 3.67)% in the Lentivirus-Foxp3 infection group. Compared with the control group and Lentivirus group, the CD86 was significantly lower in Lentivirus-Foxp3 group ($P < 0.05$).

ELISA detection IL-12, IL-10 contents

The IL-12 level in the supernatant was (200.107 ± 20.535) pg/ml in the control group, (167.080 ± 14.823) pg/ml in the Lentivirus group and (61.864 ± 21.273) pg/ml in the Lentivirus-Foxp3 group ($F = 42.880$, $P = 0.000$). Compared with the control group and Lentivirus group, the IL-12 level was significantly lower in Lentivirus-Foxp3 group ($P < 0.05$). The IL-10 level in the supernatant was (166.504 ± 31.920) pg/ml in the control group, (130.852 ± 26.962) pg/ml in the Lentivirus group and (762.421 ± 76.655) pg/ml in the Lentivirus-Foxp3 group ($F = 148.640$, $P = 0.000$). Compared with the control group and Lentivirus group, the IL-10 level was significantly higher in Lentivirus-Foxp3 group ($P < 0.05$).

SI measured by mixed lymphocyte reaction

The SI was 7.406 ± 0.090 in the control group, 7.186 ± 0.285 in the Lentivirus group, and 2.334 ± 0.298 in the Lentivirus-Foxp3 group ($F = 415.433$, $P = 0.000$). Compared with the control group and Lentivirus group, the SI was significantly lower in Lentivirus-Foxp3 group ($P < 0.05$).

Corneal allograft rejection and time to rejection

Transplant failure caused by hyphema, absence of anterior chamber and infection was observed in 8 eyes, which were excluded from the study, and extra rats were supplemented. The time to corneal rejection was 14.833 ± 1.472 d, 17.667 ± 1.366 d and 23.000 ± 2.000 d in the Group B, Group C and Group D, respectively. Compared to Group B, the time to rejection in the Group C, D and E was significantly prolonged ($P < 0.05$). The survival time of corneal graft in the Group D was longer than that in the Group C ($P < 0.05$).

Immunohistochemistry

14 days after cornea transplantation, immunohistochemistry showed no IFN- γ expression in

Foxp3 gene modified dendritic cells affect mouse corneal allograft rejection

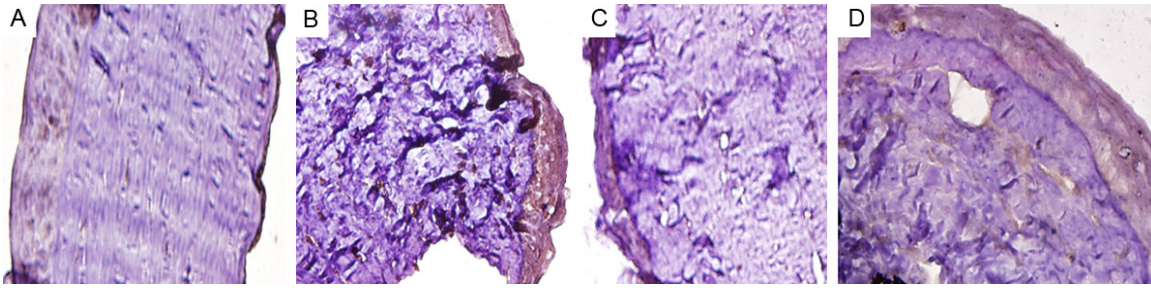


Figure 2. The expressions of IFN- γ protein in the corneal graft at 14 day after corneal transplantation in N.S treated group by immunohistochemical assay (DAB staining, $\times 400$). A. Normal cornea; B. Corneal allograft in group B; C. Corneal allograft in group C; D. Corneal allograft in group D.

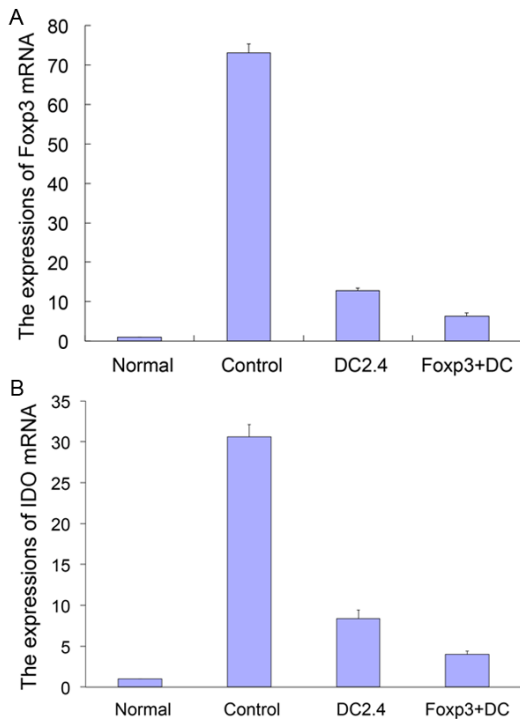


Figure 3. A. The expressions of Fxp3 mRNA in corneal allograft detected by Real-time PCR; B. The expressions of IDO mRNA in corneal allograft detected by Real-time PCR.

Group A, and markedly increased IFN- γ expression in Group B. The IFN- γ expression in Groups C and D was slightly increased when compared with Group A (**Figure 2**).

mRNA expression of Fxp3, IDO in the cornea graft after transplantation

The mRNA expression of Fxp3 in the control group, the DC group and the Fxp3 + DC group was 73.150 ± 2.140 , 12.792 ± 0.587 and 6.343 ± 0.830 , respectively, which were significantly higher than that in normal group ($P < 0.05$). Furthermore, the expression of Fxp3 in

control group was markedly higher than that in DC group and Fxp3 + DC group ($P < 0.05$). When compared with the DC group, the Fxp3 expression in the Fxp3 + DC group was markedly decreased ($P < 0.05$) (**Figure 3A**).

The mRNA expression of IDO in the control group, the DC group and the Fxp3 + DC group was 30.606 ± 1.486 , 8.394 ± 1.055 , and 3.991 ± 0.393 , respectively, which were significantly higher than that in normal group ($P < 0.05$). Furthermore, the expression of IDO in control group was markedly higher than that in DC group and Fxp3 + DC group ($P < 0.05$). When compared with the DC group, the IDO expression in the Fxp3 + DC group was markedly decreased ($P < 0.05$) (**Figure 3B**).

Discussion

Cornea transplantation has the highest success rate in transplantation field, which may be contributed to the absence of blood and lymphatic vessels and characteristic of immune privilege, and the incidence of rejection is very low in cornea transplantation. However, a lot of factors such as severe infection and chemical injury may destroy the anatomic features of cornea resulting in significantly increased post-operative rejection. Rejection in cornea transplantation is a multifactorial, complex and highly regulated process. Study [11] shows the rejection after cornea allograft transplantation is characterized by T cells mediated delayed type hypersensitivity (DTH). Further studies are required to investigate the pathogenesis of immune response after cornea transplantation and new strategies for suppression of rejection.

The characteristics of DCs determine the duality of DCs in immune and immune tolerance. Therefore, induction of immune tolerance with

Foxp3 gene modified dendritic cells affect mouse corneal allograft rejection

DCs has been a focus in the field of transplantation immunology. Studies have demonstrated that not only DCs after modification by transgenic technology but pharmacologically treated DCs can be used to induce immune tolerance [12]. DC2.4 cells are a dendritic cell line and derived from C57BL/6 mouse bone marrow cells which are transfected with GM-CSF, myc and raf genes [13]. In the present study, based on the negative immune-regulatory function of Foxp3, mouse lentivirus vectors carrying mouse Foxp3 gene (pGC-FU-Foxp3) were constructed and the Foxp3 is efficiently expressed in DC2.4 cells. Furthermore, this characteristic could be passed to daughter cells. In addition, flow cytometry showed the expressions of CD80 and CD86 were relatively low, the expression of IL-12 was decreased but that of IL-10 was increased accompanied by suppression of the proliferation of allogeneic spleen lymphocytes. In vitro studies reveal Foxp3 modified DCs can effectively suppress the proliferation of allogeneic T cells playing an important role in the induction of immune tolerance, and are the important tolerogenic DCs.

CD80 and CD86 are important costimulatory molecules and have crucial effects on the immune tolerance in transplantation. The tolerogenicity of DCs has been intensively investigated in recent years. Previous studies showed immature DCs could induce T cell tolerance due to low expressions of MHC, CD80 and CD86, and mature DCs with high expressions of these molecules could induce T cell immunity. Recent studies also showed mature DCs have tolerogenicity [14]. Researchers have used human autologous mature DCs to prepare CD4 + regulatory T cells, which could significantly suppress the mixed lymphocyte reaction [15]. In addition, DCs with high expressions of CD80 and CD86 are helpful for the amplification of regulatory T cells [16]. Therefore, there is no strict boundary in phenotype between tolerogenic DCs and immune inducing DCs. Because of the complexity of immune system and regulation network, DCs with same phenotype may exert different effects under different circumstance.

IL-12 is a critical cytokine in the immune response. IL-12 can induce the proliferation and differentiation of T lymphocytes, and mediate Th1 cell-specific immune response [17]. IL-10 is an inhibitory cytokine of immune cells. IL-10 can block the maturation of DCs and suppress the activation of T cells through down-

regulating the expressions of CD80 and CD86 on DCs. Study shows IL-10 modified DCs can effectively induce immune tolerance in transplantation [18].

In vivo experiments revealed the expressions of IFN- γ , Foxp3 and IDO were significantly increased during rejection, but markedly decreased in DCs treated mice, especially in Foxp3 + DCs treated mice. Our results showed intraperitoneal injection of Foxp3 + DCs could effectively suppress the immune response and prolong the survival time of transplants. Immunohistochemistry showed increased IFN- γ expression in the corneas of experimental group, which was not found in DC group and Foxp3 + DC group. IFN- γ is a critical cytokine secreted by Th1 cells. IFN- γ can activate macrophages and promote the expression of MHC molecules and antigen presenting playing crucial roles in the immune response in transplantation. Skurkovich et al [19] have applied IFN- γ antibody in the treatment of rejection after cornea transplantation and results show the eyesight and cornea opacity are significantly improved.

Real-time PCR showed the mRNA expressions of Foxp3 and IDO were markedly increased in experimental group, but significantly reduced in mice undergoing intraperitoneal injection of DCs and Foxp3 + DCs, which was more obvious in Foxp3 + DC group. Foxp3 is an important negative regulatory protein playing important roles in the development of CD4 + CD25 + Tr cells and the maintenance of immune functions. Indoleamine 2,3-dioxygenase (IDO) is an enzyme containing heme in the cytoplasm and an rate limiting enzyme of tryptophan metabolism. IDO can affect the functions of lymphocytes through degrading local tryptophan and plays important immunomodulatory roles in the tumor escape, maternal-fetal tolerance, chronic infection, autoimmune disease and transplantation tolerance [20, 21]. Studies have shown IDO induces immune tolerance in two ways: (1) T cells in middle G1 phase are sensitive to tryptophan and over-expression of IDO may result in insufficiency of tryptophan which compromises the proliferation and activation of T cells [22]; (2) IDO induces the proliferation of Treg cells resulting in production of IL-10 and TGF- β to suppress immune functions of T cells [23].

In the present study, real-time PCR and immunohistochemistry were employed to detect the

Foxp3 gene modified dendritic cells affect mouse corneal allograft rejection

mRNA and protein expressions of Foxp3, IDO and IFN- γ , respectively, in the cornea transplant. When compared with normal corneas, the expressions of Foxp3, IDO and IFN- γ were significantly increased in controls and higher than those in mice undergoing intraperitoneal injection of DC or Foxp3 + DC. We speculate that, during the rejection, the high expressions of Foxp3 and IDO can exert suppressive effects on Th1 cytokines including IFN- γ , which are helpful for the survival of transplant. However, the functions of IFN- γ overwhelm those of Th1 cytokines finally resulting in rejection. In mice undergoing intraperitoneal injection of DC and Foxp3 + DC, the production of Th1 cytokines (IFN- γ) is compromised and thus the expressions of IFN- γ , Foxp3 and IDO were decreased, which was more obvious in Foxp3 + DC treatment group. Therefore, the low expressions of IFN- γ , Foxp3 and IDO may be important markers for immunological tolerance.

It has been demonstrated that Foxp3 and IDO are beneficial for the transplant tolerance and the expressions of Foxp3 and IDO are increased in patients with transplant tolerance [24]. Adenovirus-mediated IDO transfection of heart results in high expression of IDO in the hearts, which suppressed the heart transplant rejection and prolonged survival time of transplant [25]. In mice undergoing cornea allograft transplantation, the survival time of transplant with high expression of IDO after transfection was significantly prolonged [26]. The IDO expression may be related to the formation of ACAID. Kynurenic acid is a product of the normal metabolism of amino acid L-tryptophan. The increased content of kynurenic acid in the corneal endothelial cells inhibits the proliferation of T cells and is beneficial for the maintenance of immune privilege of anterior chamber [27] and the survival of transplant. However, several studies also show over-expression or high expression of Foxp3 and IDO in transplant have no effects on the rejection but the expressions of both Foxp3 and IDO are markedly increased during rejection [28-30].

Taken together, our results showed in vitro Foxp3 + DCs could effectively inhibit the proliferation of allogeneic playing an important role in the immune tolerance. Therefore, Foxp3 + DCs are important tolerogenic DCs. In addition, Foxp3 + DCs were intraperitoneally injected

into mice and immunological rejection after allograft cornea transplantation was improved and the survival time of cornea graft was prolonged.

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Disclosure of conflict of interest

None.

Address correspondence to: Yi-Fei Huang, Department of Ophthalmology, Chinese People's Liberation Army (PLA) General Hospital, Beijing 100853, China. E-mail: huangyifei_h@163.com

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Foxp3 gene modified dendritic cells affect mouse corneal allograft rejection

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