Transplant Immunology 33 (2015) 192–197

Contents lists available at ScienceDirect

Transplant Immunology

journal homepage: www.elsevier.com/locate/trim

Foxp3-expressing sensitized Teff cells prolong survival of corneal allograft in corneal allograft transplantation mouse model☆

Jun Zhao [a,b,1], Zhaohui Li [c,1], Lei Wang [a], Jing Liu [a], Dajiang Wang [c], Guoling Chen [a], Qi Wang [b], Han Zhang [a,*]

[a] Department of Ophthalmology, The 2nd Hospital of Shandong University, Jinan 250031, China
[b] Department of Ophthalmology, General Hospital of Liaohe Oil Field, Panjin 124000, China
[c] The General Hospital of PLA, Beijing 100853, China

ARTICLE INFO

Article history:
Received 29 July 2015
Received in revised form 15 September 2015
Accepted 22 September 2015
Available online 28 September 2015

Keyword:
Foxp3
Treg cell
Corneal allograft
Allograft rejection
Delayed-type hypersensitivity

ABSTRACT

Objective: The study aimed to investigate whether Foxp3-expressing sensitized Teff cells could inhibit allograft rejection in corneal allograft transplantation mouse model.
Methods: Foxp3-expressing sensitized Teff cells were constructed by transfection of retroviral expression plasmid expressing Foxp3 into the sensi-Teff cells from a Balb/c mouse immunized by C57BL/6(H2b) mouse splenocytes. Balb/c mice were randomly divided into 5 groups: Four groups received tail vein injection of Foxp3-expressing sensitized Teff cells, or Foxp3-expressing Teff cells, or Treg cells or no intervention 1 day prior to corneal allograft transplantation. C57BL/6(H2b) was the donor mouse. The last group received corneal autograft transplantation. Corneal allograft survival time and percentage of $CD4^+$ T cells were detected. ELISPOT and Footpad swelling test were used to measure IL-2 and IFN-γ, and delayed-type hypersensitivity (DTH) response, respectively.
Results: Mice that had received an injection of Foxp3-expressing sensitized T cells prior to an allograft corneal transplantation, showed significantly longer survival time of corneal allograft, decreased percentage of $CD4^+$ T cells, IL-2 and IFN-γ, and alleviated footpad swelling than the mice that had received either Foxp3-Teff or Treg cells.
Conclusion: Foxp3-sensi-Teff cell treatment that prolongs corneal allograft survival in the mouse model, might partly through suppressing $CD4^+$ T cells, IL-2 and IFN-γ.

1. Introduction

Cornea is an avascular part with immune privilege and can be applied by immunosuppressive agent directly. However, corneal allograft rejection remains the predominant cause of corneal graft failure [1], accounting for 17.8% of failed corneal transplant cases [2]. It has been demonstrated that natural $CD25^+CD4^+$ regulatory T (Treg) cells and effector T (Teff) cells are critical for maintenance of immune balance. Treg cells induce immune tolerance and primarily inhibit the development of Teff cells [3]. Apoptosis of effector T cells facilitates survival of corneal allograft [4].

Accumulated evidence suggests that *in vitro* expansion of $CD4^+CD25^+$ Treg cells exhibits therapeutic effect against corneal allograft rejection [5,6]. Forkhead/winged helix transcription factor 3 $(Foxp3)^+$ is a transcription factor that is characteristically expressed by Treg cells. It has been reported that soluble CD83 (sCD83), a membrane-bound glycoprotein, prolongs the survival of corneal allograft and increases $Foxp3^+$ T cells in systemic or topical application [7]. Additionally, trichostatin A treatment could result in enhanced allosuppressive function of $CD4^+CD25^+$ Treg cells, increase expression of Foxp3 on $CD4^+CD25^+$ Treg cells and prolong survival of corneal allograft in mice [8]. A rich body of evidence has proved that Foxp3 is crucial for development, activity and function of the $CD4^+CD25^+$ Treg cells [9,10]. It has been evidenced that the capability of Treg cell to restrict allograft rejection is more largely dependent on Foxp3 expression than the amount of Treg cell [11]. Teff cells are closely associated with Treg cells and could convert into functional Treg cell with ectopic Foxp3 expression [12]. Furthermore, the successful conversion of Treg cells is primarily dependent on the expression of Foxp3 located on Treg cells [13].

Assuming that the ability of Treg cells to inhibit allograft rejection is reliant on the expression of Foxp3, then a question arises with regard to whether Foxp3-expressing Teff cells exhibit similar immunoinhibitory function to $CD4^+CD25^+$ Treg cells. To address this question, the study transfected retroviral expression plasmid expressing Foxp3 into the sensi-Teff cells which were extracted from a Balb/c mouse immunized by C57BL/6(H2b) mouse splenocytes. Then the Foxp3-expressing sensitized Teff cells were constructed and injected into a corneal transplantation mouse model. The study compared the Foxp3-expressing sensitized Teff cells and Treg cells for survival of corneal allograft,

☆ *Funding*: Research fund for The Doctoral Program of Shandong Science and Technology Department, Number: 2008BS03049.
* Corresponding author at: Department of Ophthalmology, The 2nd Hospital of Shandong University, No 247, Beiyuan Avenue, Jinan 250031, China.
E-mail address: han1_zhang@163.com (H. Zhang).
[1] Co-author: these authorscontributed equally to this work.

http://dx.doi.org/10.1016/j.trim.2015.09.003
0966-3274/

delayed-type hypersensitivity (DTH) and measurement of $CD4^+$ T cells, IL-2 and interferon-γ (IFN-γ) in corneal transplantation mouse model. The purpose of the study was to shed light on developing alternative strategies for prevention of corneal allograft rejection.

2. Materials and methods

2.1. Construction of sensi-Teff cell

Single splenic cell suspension made from a C57BL/6(H2b)mouse was subcutaneously injected in the back of a Balb/c mouse which was sacrificed two weeks later. From the spleen and lymph nodes (LNs) of a Balb/c mouse, $CD4^+CD25^+$ Treg cells or $CD4^+CD25^-$ Teff cells were isolated by column enrichment (Miltenyi Biotec, Germany) and magnetic separation with a $CD4^+CD25^+$ Treg isolation kit (Miltenyi biotec, Germany). The procedure was conducted in accordance with a previous study [14]. The Teff cells isolated from the Balb/c mouse immunized with splenocytes of C57BL/6(H2b) mouse were regarded as sensitized Teff (sensi-Teff) cells. Teff and Treg cells were also isolated from an unimmunized Balb/c mouse. Purity of the sensi-Teff cells, Teff cells and Treg cells was determined by a FACScan flow cytometer (Beckman Coulter Company, USA) [14].

2.2. Retroviral transduction for Foxp3

Retroviral transduction of Foxp3 was performed according to previous studies [15,16]. Briefly, the recombinant retroviral expression plasmid (MSCV IRES-Thy1.1/GFP encoding mouse Foxp3, Shanghai Biotechnology Company, Shanghai, China) was transfected into PA317 cells (amphotropic packaging cell line) by using FuGENE™6 transfection reagent (Roche, Switzerland). The PA317 cells were cultured in DMEM medium (Gibco, USA) for 48 h at 37 °C with 5% CO_2 in a humid atmosphere and selected with 1 mg/ml G-418. The sensi-Teff cells or Teff cells were spin-infected with retrovirus supernatant for 24 h and then cultured in new RPMI 1640 (Gibco) medium supplemented with 10% fetal calf serum (FCS, Gibco, USA). According to the manufacturer's instruction, intracellular staining for Foxp3 was performed using Fixation/Permeabilization Concentrate and Diluent (eBioscience, USA). Foxp3 expression in sensi-Teff cells or Teff cells was detected by the flow cytometry. The sensi-Teff cells or Teff cells expressing Foxp3 were defined as Foxp3-sensi-Teff cells and Foxp3-Teff cells, respectively.

2.3. Suppression of Teff cells proliferation

Foxp3-sensi-Teff cells, Foxp3-Teff cells and the sorted Treg cells were co-cultured with Teff cells at 1:1 ratio (1×10^5/100 μl/well) in 96-well plates at 37 °C with 5% CO_2 in a humid atmosphere, respectively, followed by stimulation of CD3 antibody (eBioscience Company, USA). After 72 h, cell counting kit (CCK)-8 assay (Boster Biocompany, Wuhan, China) was used to assay the Teff cell proliferation. Briefly, 10 μl CCK-8 was added to each well and cultured for another 4 h. Finally, OD value of 96-well plate was measured by microplate reader (Tecan Company, Austria). Teff cells without stimulation of CD3 antibody served as negative control. Suppression rate = (OD value of Foxp3-sensi-Teff cells − OD value of Foxp3-Teff cells) / (OD value of Foxp3-Teff cells − negative control) * 100%. Each experiment was performed in triplicate.

2.4. Corneal allograft rejection

2.4.1. Grouping

Female SPF (specific pathogen free) BALB/c mice and female C57BL/6(H2b) mice (6–8 week old, weight 18–22 g) were chosen as recipient mice and donor mice, respectively. These mice were provided by the experimental animal center of China Medical University. Donor cornea was retrieved from all the donor mice which were then sacrificed by anesthetization. All the recipient mice (n = 130) were divided into 5 groups (n = 26): Foxp3-sensi-Teff cell group which received tail vein injection of 1×10^5 Foxp3-sensi-Teff cells 1 day prior to the surgery (group A). The injection volume of cells was determined based on previous data [5]; Foxp3-Teff cell group which received tail vein injection of Foxp3-Teff cells (1×10^5) 1 day prior to the corneal allograft transplantation surgery (group B); Treg-cell group which received tail vein injection of Treg cells (1×10^5) 1 day prior to the transplantation surgery (group C); blank control group without any intervention prior to the transplantation surgery (group D); corneal autograft group without any intervention (group E). Group E received corneal autograft transplantation, while groups A–D were given corneal allograft transplantation. The animal experiment was approved by the Animal Care and Use Committee of the Second Hospital affiliated to Shandong University.

2.4.2. Corneal transplantation surgery

Murine orthotropic corneal transplantation surgery was performed as previously described [17]. Corneal stromas of recipient mice were secured with three interrupted sutures (10-0 nylon) for corneal vascularization. After 1 week, penetrating corneal transplantation was performed in recipient mice. Briefly, center cornea (2 mm in diameter) from the donor C57BL/6 mice was applied to the graft bed where a 2 mm^2 site was trephined in the central cornea of recipient mice and sutured with 8–9 interrupted 11-0 nylon sutures. For the corneal allograft group, central corneal graft was rotated 180° prior to fixation in graft bed. Finally, injection of physiological saline was used for deepening anterior chamber of the right eye in the recipient mice. The eye lids were closed by using 10-0 suture and chlortetracycline ointment was placed on the corneal surface. After 24 h, the stitches were removed from the eye lids for observation. Mice with bleeding, absence of anterior chamber, infection or Iris incarceration were excluded from the study. The corneal sutures were removed on the seventh day following surgery.

2.4.3. Observation and evaluation of corneal allograft rejection

Five mice was randomly chosen from each group, and their corneal grafts were observed under a slit-lamp microscope every day in the first two weeks after surgery and then at two days intervals for 90 days. Graft opacity was semi-quantitatively scored (0–5 +) according to a previous study [18]: 0, clear graft; 1 +, minimal superficial (non-stromal) opacity; 2 +, minimal deep (stromal) opacity; 3 +, moderate stromal opacity; 4 +, intense stromal opacity; 5 +, maximum stromal opacity. Corneal graft with opacity score ≥3 + was defined as rejected corneal graft.

2.4.4. FACS analysis of $CD4^+$ T cells

Percentage of $CD4^+$ T cells in total T cells from draining neck LNs was monitored by using FACScan flow cytometer at 10 days, 20 days, 30 days and 40 days after the transplantation surgery. At each time point, 3 mice were randomly chosen from each group and scarified for detection of $CD4^+$ T cells.

2.4.5. Enzyme-linked immunospot assay

At 21 days following transplantation, 3 mice were randomly chosen from each group and sacrificed. Cells from spleen of each mouse were measured for production of IL-2 and IFN-γ by using Enzyme-linked immunospot (ELISPOT) assay. The experiment was conducted according to previous studies [19,20]. Briefly, ELISPOT plates (ImmunoSpot; Cellular Technology, US) were coated overnight with diluted IL-2 or IFN-γ specific capture antibody (Biolegend Company, USA) in PBS solution. Cells (5×10^5) from draining LNs were seeded in these plates and cultured for 24 h. Then the cells were removed and the plated were coated with secondary biotinylated detection antibody (Biolegend Company, USA). Finally, the plate-bound second Ab was then visualized by adding streptavidin–alkaline phosphatase (DAKO, USA) and nitroblue tetrazolium (Bio-Rad, USA). A computer-assisted ELISPOT