

Pretreatment of Rapamycin Before Allogenic Corneal Transplant Promotes Graft Survival Through Increasing CD4⁺CD25⁺Foxp3⁺ Regulatory T Cells

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Abstract

Objectives: To evaluate the effect of rapamycin pretreatment before allogenic corneal transplant on CD4⁺CD25⁺Foxp3⁺T regulatory cells (Treg) in recipient mice, and analyze its correlation with graft survival.

Materials and Methods: Balb/c mice were intraperitoneally injected with rapamycin or control solution for 2 weeks. They then underwent a corneal transplant with C57/BL6 serving as the donor. Graft status was assessed twice a week. Recipient mice were killed 14 days after surgery, and the percentage of CD4⁺CD25⁺Foxp3⁺Treg in peripheral blood, spleen, and draining lymph nodes was analyzed by flow cytometry. Moreover, CD4⁺CD25⁺T cells in corneal grafts and conjunctiva were identified, and expression of Foxp3 mRNA in the grafts was tested. Additionally, the concentration of IL-10 and TGF-β1 in serum and aqueous humor was measured.

Results: Pretreatment of rapamycin significantly enhanced the percentage of CD4⁺CD25⁺Foxp3⁺Treg in peripheral blood and draining lymph nodes, preoperatively and postoperatively, which had significant negative correlation with graft opacity and neovascularization. Moreover, rapamycin pretreatment led to a larger number of CD4⁺CD25⁺T cells infiltrating in corneal grafts and conjunctiva, increased expression of Foxp3 mRNA

in grafts, and elevated concentration of TGF-β1 in aqueous humor.

Conclusions: Pretreatment with rapamycin for 14 days before an allogenic corneal transplant enhances the percentage of CD4⁺CD25⁺Foxp3⁺Treg cells in peripheral blood, draining lymph nodes, and grafts, thereby inhibiting graft rejection.

Key words: Rapamycin, Regulatory T cells, Corneal transplant

Corneal disease, one of the leading causes of blindness in China,¹ requires a corneal transplant to restore visual function in most cases.² Although immune privilege of corneal allografts endows a high success rate of corneal transplant than other solid-organ transplants, immunologic rejection remains a major cause of graft failure after corneal transplant.^{3,4}

Regulatory CD4⁺CD25⁺Foxp3⁺T cells (Treg), an important regulator in maintaining immune homeostasis, play a crucial role in autoimmune diseases and tumors, and protect individuals from graft rejection.⁵ However, Treg cells are present in low numbers under normal conditions, reported to be 5% to 10% of CD4⁺T cells in mice and human blood, and have an anergic phenotype.⁶ Therefore, enhancement of activated Treg cells may protect individuals from autoimmune diseases and graft rejection, just as previous studies have revealed.⁷⁻¹⁰

Rapamycin, a novel macrolide immunosuppressive drug, has been reported to prevent clinical allograft rejections including corneal allografts.¹¹ Moreover, rapamycin allows expansion of CD4⁺CD25⁺Foxp3⁺ Treg in vitro and in vivo,¹²⁻¹⁴ and CD4⁺CD25⁺Foxp3⁺ Treg converted by rapamycin has more-potent regulatory abilities.¹⁵ It has been shown that adoptive transfer of CD4⁺CD25⁺Foxp3⁺ Treg expanded by rapamycin in vitro is capable of

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promoting tolerance to allogenic grafts.¹⁶ However, the ability of enhanced CD4⁺CD25⁺Foxp3⁺ Tregs, induced by in vivo rapamycin administration before allotransplant, to prohibit rejection and promote graft acceptance, remains unclear. Hence, we sought to explore whether in vivo enhancement of CD4⁺CD25⁺Foxp3⁺ Treg, induced by rapamycin pretreatment before allogenic corneal transplant, could protect the corneal graft in recipient mice after surgery.

Materials and Methods

Mice

Forty Balb/c (H-2d) and 20 C57/BL6 (H-2b) female mice (weighing 20-24 g, 6-8 wk old) were purchased from the Department of Laboratory Animal, Fudan University. All mice were housed in a specific pathogen-free environment. All experimental manipulations were undertaken in accordance with the Institutional Guidelines for the Care and Use of Laboratory Animals and ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Rapamycin treatment

Rapamycin in powder form (Wyeth Ayerst-Research, Princeton, NJ, USA [now part of Pfizer]) was dissolved in the vehicle containing sodium CMC (C-5013 high viscosity; Sigma-Aldrich Corp. St. Louis, MO, USA) and polysorbate 80 (P-8074, Sigma-Aldrich). Rapamycin solution was stored at 4°C in the dark according to the manufacturer's instruction. The control solution only included 0.2% CMC and 0.25% polysorbate 80.

Before undergoing a corneal transplant, Balb/c mice were randomly divided into 2 groups, with each group containing 20 recipient mice. They then were intraperitoneally injected with either rapamycin 1.5 mg/kg/d or the same volume of a control solution. The injection was performed once per day and lasted for 2 weeks. Then 5 mice in each group were killed, and peripheral blood, spleen, and draining lymph nodes were collected for flow cytometric analysis. The rest of the mice underwent an allogenic corneal transplant.

Corneal transplant (penetrating keratoplasty)

Allogenic penetrating keratoplasty was performed as previously reported.¹⁷ In brief, C57/BL6 mice were killed by cervical dislocation, and a 2-mm diameter trephine was used to obtain the cornea as the donor.

Rapamycin or control solution pretreated Balb/c mice, with the right eyes serving as the recipients, were anesthetized with 100 mg/kg ketamine hydrochloride and 5 mg/kg diazepam. After dilating the pupil by administering 0.25% tropicamide eye drops, a 1.5-mm-diameter trephine was used to cut the recipient cornea at the depth of 80% to 90%, and then paracentesis was carefully done with a 1-mL needle to avoid damaging the lens. Viscoelastic material containing 3% sodium hyaluronate (Healon; Advanced Medical Optics, Santa Ana, CA, USA) was injected immediately to maintain the anterior chamber. Then, the recipient cornea was cut with a scissors, and the donor cornea was fixed to the recipient's bed with 8 interrupted 11-0 nylon sutures. Erythromycin eye ointment was administered in the conjunctival sac after surgery, and the eyelids were sutured with 8-0 mattress sutures. The eyelid sutures were removed 3 days after surgery; neither systematic nor topical immunosuppressive drugs were applied.

Assessment of the grafts

Three, 7, 10, and 14 days after surgery, all recipient mice were examined under a slit lamp biomicroscope, and digital photographs of the cornea were taken using a Canon 8-megapixel digital camera attached to the slit lamp biomicroscope. Transparency of the graft and degree of neovascularization in the graft and host tissue were recorded and scored to assess graft rejection according to the criteria listed in Table 1, which was put forward by previous researchers.¹⁷⁻¹⁸ Mice with infection, hyphema, and cataracts were excluded, and the same quantity of recipient mice were recruited.

Flow cytometric analysis

Fifteen days after surgery, peripheral blood from the recipient mice was collected in heparinized tubes, and the spleen and draining lymph nodes were obtained after the animals were killed. Single cell suspension of the spleen and lymph nodes were prepared through compression with the plunger of a 3-mL syringe. Subsequently, cells were centrifuged and resuspended in appropriate volume of flow cytometry staining buffer to adjust the final cell concentration to 2×10^7 /mL. Then, cells were stained with a mouse Treg staining kit (eBioscience, Inc., 88-8111, San Diego, CA, USA) according to the manufacturer's instructions, and 3-color flow cytometry was performed. Briefly, either the peripheral blood or cell

Table 1. Clinical Scoring Criteria for Orthotopic Corneal Grafts

Score	Clinical Appearance
Opacity	
0	Clear graft
1	Minimal, superficial opacity not involving corneal stroma
2	Minimal to moderate opacity including corneal stroma
3	Moderate stromal opacity, where pupil is visible and the iris is obscured
4	Intense stromal opacity, where only outline of the pupil is visible
5	Severe stromal opacity, where the pupil is not visible
Neovascularization	
0	No neovascularization
1	Vessels in < 50% of the recipient graft bed only
2	Vessels in > 50% of the recipient graft bed only
3	Vessels in < 50% of the recipient-graft border
4	Vessels in > 50% of the recipient-graft border
5	Vessels in < 50% of the peripheral graft stroma
6	Vessels in > 50% of the peripheral graft stroma
7	Vessels in < 50% of the central graft stroma
8	Vessels in > 50% of the central graft stroma

suspension was incubated with anti-mouse CD4-FITC (RM4-5) and anti-mouse CD25-APC (PC61.5) at 4°C for at least 30 minutes in the dark. The cells then were rinsed with precooling flow cytometry staining buffer, followed by treatment with 1 mL of fixation/permeabilization working solution for 30 minutes in the dark. After washing the cells twice with the permeabilization buffer, the cells were resuspended in 500 µL of permeabilization buffer, and anti-mouse Foxp3-PE (FJK-16s) was added. Cells were then incubated at 4°C for another 30 minutes in the dark, followed by 2 more rinses with the permeabilization buffer. Finally, cells were resuspended with the flow cytometry staining buffer, and analyzed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) and CellQuest software.

Immunofluorescence staining

After killing recipient mice, the eyeballs were enucleated and fixed in the mixture of 40% formaldehyde, 40% glacial acetic acid, and 95% ethanol for 24 hours, then dehydrated in graded ethanol solutions and embedded in paraffin wax. The embedded tissue was cut to 4- to 6-µm-thick sections. To perform immunofluorescence staining, the sections were firstly deparaffinized in xylene using 3 changes for 5 minutes each, and then hydrated gradually through graded alcohols: washed in 100% ethanol twice for 10 minutes each, then 95% ethanol twice for 10 minutes each. Antigen retrieval was performed by heating the slides in 10 mM sodium citrate buffer, pH 6.0, at 95°C for 5 minutes, and then cooling them in the

buffer for approximately 20 minutes until room temperature. After this, slices were washed with phosphate buffered saline 3 times, and excess liquid was aspirated. After several blocking steps, the sections were incubated with 1:100 goat anti-mouse CD4 (Santa-Cruz C-18, Santa Cruz, CA, USA) and rat anti-mouse CD25 (PC61.5, eBioscience) for 1 hour at 37°C and then overnight at 4°C. Slices then were washed with phosphate buffered saline 3 times for 5 minutes each and incubated with Alexa Fluor 594 rabbit anti-goat IgG (HH-A11080, Invitrogen Corporation, Carlsbad, CA, USA) and Alexa Fluor 488 rabbit anti-rat IgG (HH-A21210, Invitrogen) for 2 hours. Finally, sections were mounted with glycerol and examined under laser scanning confocal microscopy (TCS SP2, Leica, Germany).

Real-time polymerase chain reaction of Foxp3 in the graft

After killing recipient mice and enucleating the eyeballs, each cornea was dissected from the limbus, and expression of the Foxp3 gene in the corneal tissue was analyzed using quantitative real-time polymerase chain reaction. Total RNA was extracted from homogenized dissected corneal grafts using the Qiagen RNeasy miniKit (Qiagen, Tokyo, Japan), according to the manufacturer's instructions. Reverse transcription to cDNA was performed using TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA, USA). The sequence of forward and reverse primer was 5' CTATGCCACCCTTATCCGA 3' and 5' TCCTCTTCTTGCGAAACTCA 3'. Mouse β-actin was used as an endogenous control. Relative quantification assays for Foxp3 gene expression were performed using a StepOnePlus real-time polymerase chain reaction system (Applied Biosystems). For each example, the threshold cycle (CT) value of Foxp3 was normalized using the formula $\Delta CT = CT^{Foxp3} - CT^{\beta-actin}$. The mean ΔCT was determined, and the relative foxp3 mRNA expression was calculated with $2^{-\Delta CT}$.

Enzyme-linked immunosorbent assay

Peripheral blood and aqueous humor were collected before killing recipient mice, and enzyme-linked immunosorbent assay was performed to test expression of TGF-β1 and IL-10 in the serum and aqueous humor, using enzyme-linked immunosorbent assay kits (81-BMS614/2 and 81-BMS608/3; eBioscience San Diego, CA, USA).

Statistical analyses

Data are presented as means ± SD. The *t* test and Kruskal-Wallis test were used to analyze the data. The Pearson correlation analysis was performed to explore the relation between the level of CD4⁺CD25⁺Foxp3⁺Tregs and graft survival. All tests were considered statistically significant at *P* < .05. Statistical analyses were performed with SPSS software (SPSS: An IBM Company, version 13.0, IBM Corporation, Armonk, New York, USA).

Results

Effect of rapamycin treatment on CD4⁺CD25⁺Foxp3⁺Tregs

Pretreatment of rapamycin significantly increased the level of CD4⁺CD25⁺Foxp3⁺Tregs in peripheral blood and draining lymph nodes before allogenic penetrating keratoplasty was performed, just as Figure 1 shows. Moreover, even at 14 days after surgery, the level of CD4⁺CD25⁺Foxp3⁺Tregs in peripheral blood and draining lymph nodes remained significantly higher in the rapamycin-treated group.

The score of opacity and neovascularization

Figure 2 reveals the scores of graft opacity and neovascularization, indicating that scores in the rapamycin group were significantly lower than those

of the control group, except 3 days after surgery. Further correlation analysis demonstrated that the scores of graft opacity and neovascularization had a significant negative correlation with postoperative level of CD4⁺CD25⁺Foxp3⁺Tregs in the peripheral blood and draining lymph nodes (opacity: $\rho = -0.762$ and -0.662 , neovascularization: $\rho = -0.720$ and -0.641 ; *P* < .05).

CD4⁺CD25⁺Foxp3⁺Treg in the graft

In the rapamycin-treated group, more CD4⁺CD25⁺T cells were found to infiltrate in the corneal grafts and conjunctiva (Figures 3A and B). Moreover, the expression level of Foxp3 gene was significantly elevated in grafts from recipient mice treated with rapamycin, compared with controls ($2^{-\Delta CT}$ value: 0.072 ± 0.010 vs 0.045 ± 0.016 ; *P* < .05).

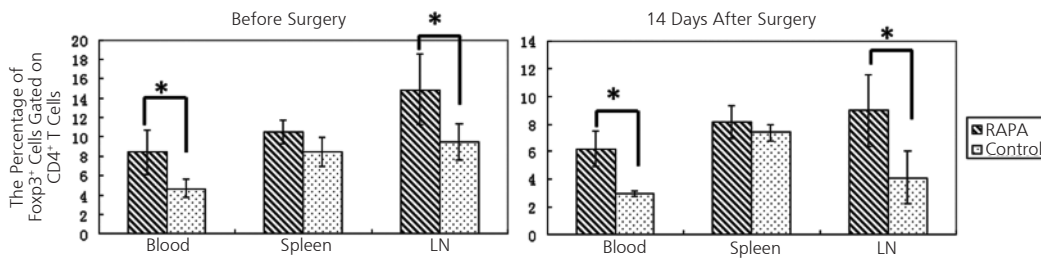
TGF- β 1 and IL-10 level in serum and aqueous humor

Compared with controls, rapamycin treatment caused significant increase of TGF- β 1 in aqueous humor (Figure 4). However, the treatment did not influence the concentration of IL-10.

Discussion

Although Tregs have strong immunosuppressive potency, they are present in low numbers in a normal

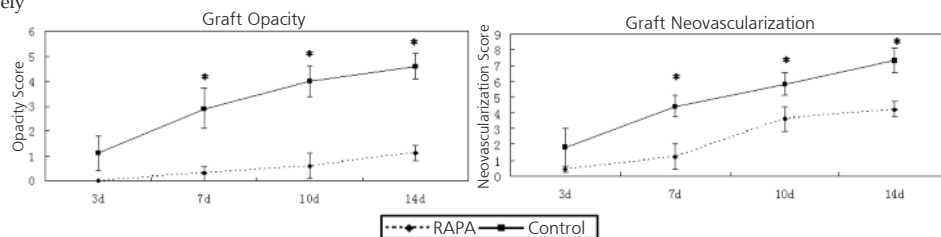
Figure 1. The Percentage of CD4⁺CD25⁺Foxp3⁺Treg in Blood, Spleen, and Draining Lymph Nodes



Abbreviations: LN, lymph nodes; RAPA, rapamycin

Administration of rapamycin before allogenic penetrating keratoplasty enhances expression of CD4⁺CD25⁺Foxp3⁺Treg. Even at 14 days after surgery, Treg levels in rapamycin-pretreated groups were significantly higher. The numbers represent percentages of Foxp3-expressing cells gated on live CD4⁺T cells. Representative data are shown of 3 experiments performed. Error bars refer to the SEM of 3 independent experiments. **P* < .05.

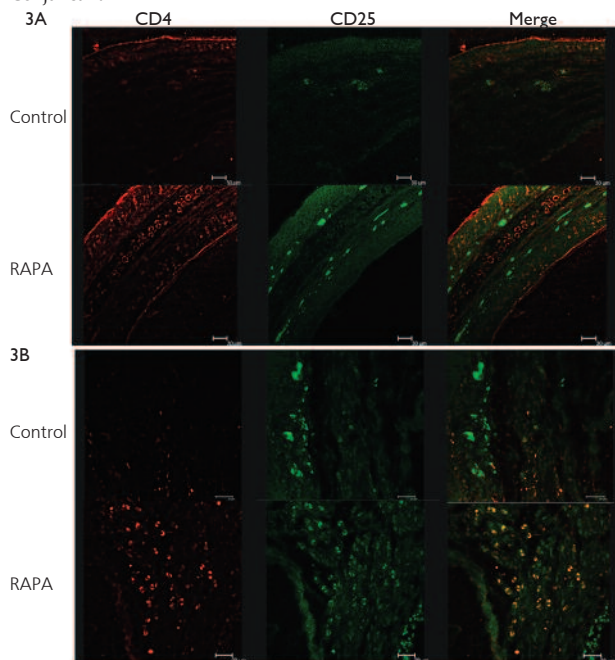
Figure 2. Pretreatment of Rapamycin Could Reduce the Score of Graft Opacity and Neovascularization After Subsequent Allogenic Penetrating Keratoplasty, Except 3 Days Postoperatively



Abbreviations: RAPA, rapamycin

**P* < .05.

Figure 3. Immunostaining of CD4⁺CD25⁺ T Cells in Corneal Grafts and Conjunctiva



Abbreviations: RAPA, rapamycin

In mice pretreated with control solution, few CD4⁺CD25⁺ T cells were found to infiltrate in corneal grafts (3A, top line) or conjunctiva (3B, top line). In contrast, pretreatment of rapamycin could significantly increase the number of CD4⁺CD25⁺ T cells in both corneal grafts (3A, bottom line) and conjunctiva (3B, bottom line).

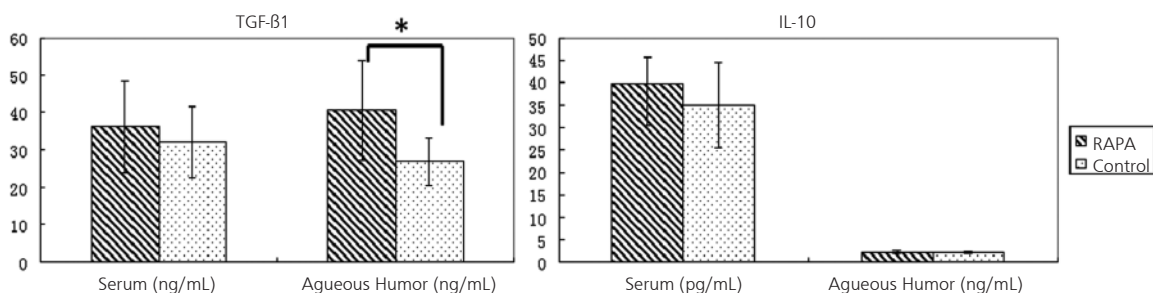
host and have an anergic phenotype, because of which it is difficult to harness their tolerogenic potential to treat autoimmunity and transplant rejection. So, a safe and easy method to expand Treg cells before allogeneic organ transplant is not only crucial to protect the allograft and achieve graft acceptance, but also beneficial in reducing the dosage and adverse effects of postoperative antirejection drugs.

The current study found that *in vivo* administration of rapamycin for 14 days before allotransplant expanded CD4⁺CD25⁺Foxp3⁺Treg

population in the peripheral blood and draining lymph nodes, which agrees with previous reports.^{15,19} Rapamycin has been shown to allow expansion of functional CD4⁺CD25⁺Foxp3⁺Treg, *in vivo* and *in vitro*.¹²⁻¹⁴ The molecular mechanism of immunosuppression mediated by rapamycin was that it bound to FK506 binding protein 12, inhibited mammalian target of rapamycin (mTOR), and in turn, hindered formation of the mTOR complex (mTORC).²⁰⁻²¹ Inhibition of mTOR could drive naïve CD4⁺ T cells to differentiate into Foxp3⁺ Tregs, with the participation of histone H3K4me2 and 3-methylation near the Foxp3 transcriptional start site.²²⁻²³ Moreover, Foxp3 expression induced pim2 expression in Tregs, which allowed for a selective growth advantage of Treg in the presence of rapamycin.²⁴

It is noteworthy that the elevated expression of CD4⁺CD25⁺Foxp3⁺Treg induced by preoperative treatment of rapamycin sustained at least 2 weeks in peripheral blood, draining lymph nodes, and corneal tissue even after the withdrawal of rapamycin, as shown in the present study. It has been reported that *in vivo* rapamycin administration contributed to selective activation and expansion of CD4⁺CD25⁺Foxp3⁺Treg, not only in peripheral blood, but also, in secondary lymphoid organs and grafts, both of which merit graft survival.^{15,25-26} Therefore, it is reasonable to deduce that the increased numbers of CD4⁺CD25⁺Foxp3⁺Treg found in draining lymph nodes and corneal grafts play a pivotal role in inhibiting graft rejection after allogeneic keratoplasty. Moreover, previous research has observed that the number of Tregs increased shortly after rapamycin therapy was stopped, and then decreased gradually.²⁷ This might be a possible explanation for the finding that the grafts in rapamycin-pretreated group

Figure 4. Comparison of TGF-β1 and IL-10 Levels



Abbreviations: IL, interleukin; RAPA, rapamycin; TGF, transforming growth factor

Comparison of the level of TGF-β1 and IL-10 between rapamycin-pretreated mice and controls reveals a significant increase of TGF-β1 in aqueous humor in rapamycin-pretreated ones. **P* < .05.

presented little evidence of rejection, even when no immunosuppressive drugs were used postoperatively.

The current study shows the concentration of TGF- β 1 in aqueous humor significantly increases in recipient mice pretreated with rapamycin. It has been demonstrated that Treg cells use multiple means to restrain inflammation including secretion of immunosuppressive cytokines such as TGF- β 1. Various studies have reported that after solid organ transplant, elevated content of TGF- β 1 was found among patients with grafts accepted or without acute rejection episodes.²⁸⁻³⁰ On one hand, TGF- β 1 has been shown to be a stabilizer of Foxp3 expression, maintaining the effector function of CD4⁺Foxp3⁺ Treg cells, and ultimately sustaining Treg peripheral homeostasis. On the other hand, TGF- β 1 is involved in Treg suppressive activity, especially during the induction of allograft tolerance.³¹ A significant positive correlation between active suppression and TGF- β 1 in peripheral blood has been identified in renal allograft recipients.³² Our study unveiled a similar finding that the level of TGF- β 1 in aqueous humor was significantly higher in ones with unrejected grafts. Considering the fact that the cytokines and cells in aqueous humor, rather than those in serum, play a predominant role in mediating immunologic reaction to the corneal graft and determining its ultimate outcome, our findings confirm the immunomodulatory effect of TGF- β 1 produced by Treg- favored allograft acceptance.

Unexpectedly, we found levels of IL-10 in serum and aqueous humor were similar between rapamycin-pretreated recipient mice and controls. It usually has been accepted that IL-10 produced by Tregs plays a role in immunoregulation and immunosuppression.³³ However, the effect of IL-10 remains controversial. Pyzik and associates and Brandenburg and associates both have demonstrated that an elevated number of Tregs is accompanied by increased IL-10 levels in vivo, and IL-10 neutralization abrogates the suppressive effect, in vivo and in vitro.^{34,35} Moreover, IL-10 has been shown to potentiate TGF- β 1 effects.³⁶ On the contrary, Strauss and associates reported that neither fresh CD4⁺CD25⁺ T cells nor R0 and R1 T cells after in vitro expansion expressed IL-10 as measured by flow cytometry.³⁷ Pillai and associates also reported that IL-10/IL-35 double-deficient Tregs were fully functional in vitro and in vivo.³⁸ Combining these

findings together, we suppose that Tregs induced by rapamycin pretreatment probably exerted immunosuppressive effects through cross-regulatory pathways other than IL-10.

The major shortcoming of this study was the relatively short follow-up after keratoplasty because it is a pilot study. Graft outcomes after 2 weeks or even longer remained unclear. Furthermore, how long could the population of Treg cells sustain a level sufficient enough to inhibit rejection, which was activated by pretreatment of rapamycin for 2 weeks, until it was insufficient to maintain the tolerance of grafts? Is repeated therapy required to maintain the graft transparency, and how about the injection frequencies? These issues should be addressed by further investigations.

It also should be considered that although treatment of rapamycin after high-risk keratoplasty has been used in a small number of patients and its efficacy has been proved, the adverse effects remain undetermined thoroughly.³⁹ Likewise, the dosage, administration route, and safety of rapamycin pretreatment should be clarified before the therapeutic protocol is applied in human beings to prevent immunologic rejection after penetrating keratoplasty.

In summary, pretreatment of rapamycin for 14 days before an allogenic corneal transplant enhances the percentage of CD4⁺CD25⁺Foxp3⁺Treg cells in peripheral blood, draining lymph nodes, and grafts, thereby inhibiting graft rejection. Our findings suggest that the elevated level of CD4⁺CD25⁺Foxp3⁺Treg cells before allotransplant produce a beneficial effect on the induction of immunologic tolerance and reduce the dosage and adverse effects of anti-rejection drugs after transplantation. This protocol provides a new direction for the treatment of immunologic rejection.

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