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Effect of Rapamycin and Interleukin-2 on Regulatory $CD4^+CD25^+Foxp3^+$ T Cells in Mice After Allogenic Corneal Transplantation

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ABSTRACT

Background. We explored the effect of rapamycin (RAPA) and interleukin (IL)-2 on regulatory $CD4^+CD25^+Foxp3^+$ T cells (Treg) in recipient mice after allogenic corneal transplantation and analyzed its correlation with graft outcome.

Methods. Allogenic corneal transplantation was performed using C57/BL6 mice as donors and Balb/c mice as recipients. RAPA, IL-2, and RAPA + IL-2 (mixed group) were administered to recipient mice, with three dosages for each therapeutic protocol. The graft status was assessed twice per week. The percentage of $CD4^+CD25^+Foxp3^+$ Treg in the peripheral blood, spleen, and draining lymph nodes was analyzed. The expression of Foxp3 mRNA in grafts was tested, and the concentration of IL-10 and transforming growth factor (TGF)-β1 in serum and aqueous humor was measured.

Results. The lowest scores of graft neovascularization and opacity were mainly found in mixed groups. The percentage of $CD4^+CD25^+Foxp3^+$ Tregs in blood was increased significantly in mice treated with either high-dose RAPA or high-dose IL-2, and a synergistic effect was found in mixed high-dose group. So were the Tregs in either spleen or draining lymph nodes. However, such effects were weakened with decreased dosage. Foxp3 gene expression in grafts was elevated significantly in the recipients treated with median dosage of RAPA, IL-2, and mixed agents. The concentration of IL-10 in serum and aqueous humor was increased significantly in mice with mixed- high-dose treatment. Mixed treatments also enhanced TGF-β1 level in serum and aqueous humor, except those receiving low dosage.

Conclusion. In vivo administration of RAPA prohibited graft rejection after allogenic penetrating keratoplasty through expansion of $CD4^+CD25^+Foxp3^+$ Tregs. Simultaneous treatment of IL-2 enabled further elevation of Tregs. However, the synergistic effect was dosage-dependent, being the most potent at high dosage. The protocol may be beneficial to induce transplantation tolerance.

CORNEAL DISEASE is the one of the leading causes of blindness in China,[1] the majority of which requires corneal transplantation to restore visual function.[2] Although the immune privilege of corneal allografts has endowed corneal transplantation with a higher success rate than other solid organ transplantations, immunologic rejection is still the major cause of graft failure after penetrating keratoplasty.[3]

Regulatory T cells, (Treg) also called suppressor T cells, comprising approximately 5% to 10% of the peripheral $CD4^+$ T cells in humans and mice, are the most important regulators to maintaining immune homeostasis. They are divided into two groups based on their origin and functionality, named natural Treg and induced Treg (iTreg) cells.[4] Both Treg populations are characterized by high-level

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expression of the surface interleukin (IL)-2 receptor α chain (IL-2Rα, CD25) and intracellular expression of a master switch transcription factor called the forkhead box protein P3 (FoxP3), and they have been indistinguishable by their surface markers until now.[5] Enhancing activated $CD4^+CD25^+Foxp3^+$ Treg cells may protect individuals from autoimmune diseases and graft rejection, while suppressing $CD4^+CD25^+Foxp3^+$ Treg cell activity may increase the potential occurrence of graft rejection, autoimmune diseases, and tumors.[6–9]

Rapamycin (RAPA), a novel macrolide immunosuppressive drug, has been used widely to prevent clinical allograft rejections, including corneal allografts.[10] According to prior research, RAPA allows an expansion of $CD4^+CD25^+Foxp3^+$ Treg cells both in vitro and in vivo,[11–14] including the recipient after liver or cardiac transplantation.[15,16] The molecular mechanism of immunosuppression mediated by RAPA is that it binds to FK506 binding protein 12 (FKBP12) and the formed complex inhibits the mammalian target's function, which, in turn, prohibits protein phosphorylation and cell cycle progression.[17] Moreover, previous research indicates that RAPA does not inhibit IL-2 production from antigen-induced T-cell activation.[18] There is significant evidence that IL-2 is essential for the peripheral homeostasis of $CD4^+CD25^+Foxp3^+$ Treg cells and can selectively promote Treg suppressive activity from both in vitro and in vivo studies.[19,20] A recent in vitro investigation demonstrated that $CD4^+CD25^+$ cells expanded with the combination of RAPA and IL-2 had more immunosuppressive ability than cells expanded with IL-2 solely.[21]

However, to the best of our knowledge, no in vivo studies have been published about the effect of RAPA and IL-2 on $CD4^+CD25^+Foxp3^+$ Treg cells in the recipient after solid organ transplantation. Hence, we performed the current study to explore the effect of the rapamycin, IL-2, and the combination of the two agents on the expression of $CD4^+CD25^+Foxp3^+$ Treg cells in recipient mice after allogenic penetrating keratoplasty and to analyze its correlation with graft survival.

MATERIALS AND METHODS

Mice

One hundred fifty Balb/c (H-2d) and 75 C57/BL6 (H-2b) female mice (weighing 20–24 g, 6–8 weeks 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 the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research.

Allogenic Penetrating Keratoplasty

The surgical procedure was performed as previously reported.[22] In brief, C57/BL6 mice were sacrificed by cervical dislocation, and a 2-mm-diameter trephine was used to obtain the cornea from the donor. Balb/c mice, with the right eyes serving as the recipients, were anesthetized with 100 mg/kg of ketamine hydrochloride and 5 mg/kg of 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, Calif, USA) was injected immediately to maintain the anterior chamber. Then, the recipient cornea was cut with scissors and the donor cornea was fixed to the recipient's bed with eight interrupted 11-0 nylon sutures. Erythromycin eye ointment was administered in the conjunctival sac after the surgery, and then the eyelids were sutured with an 8-0 mattress sutures. The eyelids sutures were removed at day 3 postoperatively.

RAPA and IL-2 Treatment

RAPA in powder form (Wyeth-Ayerst, Princeton, NJ, USA) was dissolved in the vehicle containing sodium carboxymethylcellulose (C-5013 high viscosity, Sigma-Aldrich) and polysorbate 80 (P-8074, Sigma-Aldrich). The RAPA 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. IL-2 (Prepro-Tech, NJ, USA) was diluted with steril deionized water to the intended concentration.

After the surgery, the Balb/c mice were randomly divided into 10 groups and each group contained 15 recipient mice. Then, they were intraperitoneally injected with one of three dosages of RAPA solution (RAPA-high: 1.5 mg/kg/d; RAPA-median: 0.75 mg/kg/d; RAPA-low: 0.375 mg/kg/d), three dosages of IL-2 solution (IL-2-high: 15,000 IU/kg/d; IL-2-median: 7500 IU/kg/d; IL-2-low: 3750 IU/kg/d), three combination of RAPA and IL-2 at different dosages (mixed-high: RAPA 1.5 mg/kg/d + IL-2 15,000 IU/kg/d; mixed-median: RAPA 0.75 mg/kg/d + IL-2 7500 IU/kg/d; mixed-low: RAPA 0.375 mg/kg/d + IL-2 3750 IU/Kg/d), and the control solution, respectively. The injection was performed once per day and lasted for 2 weeks.

Assessment of the Grafts

Three, 7, 10, and 14 days after surgery, all recipient mice were examined under the slit-lamp biomicroscope, and digital photographs of the cornea were taken using a Canon 8-megapixel digital camera attached to the slit-lamp biomicroscope. The transparency of the graft and the formation of neovascularization in the graft and host tissue were recorded and scored to determine graft acceptance or rejection according to the previous literature.[22,23] Mice with infection, hyphema, and cataract were excluded and the same quantity of recipient mice was recruited.

Flow Cytometric Analysis

Fifteen days after surgery, peripheral blood from five recipient mice in each group was collected in heperanized tubes, and the spleen and draining lymph nodes were also obtained after the animals were sacrificed. Single-cell suspension of the spleen and lymph nodes were prepared through compression with the plunger of a 3-mL syringe. Subsequently, the cells were centrifuged and resuspended in an 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, 88-8111, Calif, USA) according to the manufacturer's instruction and three-color flow cytometry was performed. Briefly, either the