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AN EXPERIMENTAL RESEARCH OF THE EFFECTS OF RAPAMYCIN ON THE IN VITRO PROLIFERATION AND GROWTH OF TREGS IN MICE BY PROMOTING TGF-B SECRETION

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Abstract : Objective: In vitro co-culture of CD4+CD25+Tregs with Rapamycin or Cyclosporine A was observed on the proliferation of CD4+CD25+Tregs and expression of Foxp3 and TGF-β, in order to analyze the mechanism by which Rapamycin promotes TGF-β to secrete and induce in vitro differentiation and proliferation of regulatory T cells (Tregs). Methods: The mononuclear cell was isolated from a C57BL/6 mouse spleen obtained under the sterile conditions; CD4+CD25+Tregs were sorted by immunomagnetic beads, which were divided into the blank control group, Rapamycin group, and Cyclosporine A group to conduct 96 hours of co-culture. CD4+CD25+Tregs were detected with a up-flow cytometry. The level of expression and secretion of FoxP3+ and TGF-βmRNA of CD4+CD25+Tregs treated by Rapamycin and Cyclosporin A were detected and analyzed with reverse transcription-polymerase chain reaction (RT-PCR) and enzyme linked immunosorbent assay (ELISA). The expression of Smad proteins, which is the important activated molecules of the TGF-β signal pathway, was analyzed by Western blot,and observed the effects on the proliferation of CD4+CD25+FoxP3+Tregs. TGF-β neutralizing antibodies were used to further confirm the significance of Rapamycin on the promotion of differentiation and proliferation of CD4+CD25+FoxP3+Tregs. Results: Compared with that in the control group, the proportion of CD4+CD25+Tregs, decreasing significantly in CD4+T cells (3.72% vs 7.42%, $p<0.01$)in the Cyclosporine group, and obviously increased (11.47% vs 7.42%, $p<0.01$) in the Rapamycin group; the expression of T cells and Foxp3 mRNA of Rapamycin group was significantly higher than that in the Cyclosporine group and control group (all $P<0.01$); the expression of Foxp3 mRNA was lower markedly in the Cyclosporine A group compared with that in the control group ($P<0.05$). Conclusions: Rapamycin can promote the proliferation and growth of CD4+CD25+Tregs by in vitro culture, while Cyclosporin A can inhibit proliferation and growth of CD4+CD25+Tregs in vitro culture; Foxp3 and TGF-β are related positively to CD4+CD25+Tregs by the in vitro experiment. Rapamycin can promote the proliferation of CD4+CD25+ FoxP3+Tregs by inducing the secretion and expression of TGF-β.

Key words: Regulatory T cells; Mouse; Rapamycin; Cyclosporine A; TGF-β; Foxp3

1. INTRODUCTION

The degree of allograft rejection or transplantation tolerance depends on the degree of balance between effector cells and Tregs. As has gradually become the focus and direction on the research in recent years, immunosuppressive agents are used to promote the regulation of Tregs to effector T cells in order to effectively prevent rejection. It is reported that transforming growth factor-β (TGF-β) signal is the key to the functional activation of Tregs. The article by Ingrid et al. has demonstrated that Rapamycin can stimulate the production of TGF-β, but it is not clear whether the effect of Rapamycin on Tregs is associated with the induced TGF-β. Therefore CD4+ cells isolated in vitro were co-cultured with Cyclosporine A or Rapamycin etc.to observe proportion of Tregs and the secretion level of TGF-β, to understand the proliferation of Tregs and the expression of Foxp3, and to research whether Rapamycin can induce the proliferation of Tregs and play a corresponding role in immune regulation by promoting the production of TGF-β.Meanwhile, the effects of Rapamycin and Cyclosporine A on the differentiation of Tregs mediated by TGF-β were compared in vitro and in vivo.All of these will provide an experimental basis for designing the therapeutic regimen of immunosuppressive agents after cardiac transplantation in clinical.

2. METHODS:

Animals

Male, specific pathogen free (SPF), C57BL/6 (H-2Kb) and BALB/C (H-2Kd) mice aged 8-12 weeks, weighing about 20kg, provided by Shanghai Bikai Experimental Animal Co. Ltd., fed in different cages in an animal room of the immune teaching and research section of the Second Military Medical University, and experimented and fed on the basis of relevant experimental regulations for animals.

Materials and Reagents

Rapamycin (5 mg/5ml): purchased by the U. S. Wyeth Pharmaceutical Co. Ltd; Cyclosporined A (50, mg/5 mL): produced by Swiss Novartis; Trizol reagent: American Invitrogen Corporation; Chloroform: Sinopharm Chemical Reagent Co. Ltd; Agrose: CUSABIO Biotech CO. Ltd; all cells were cultured under 37ºC, 5%CO2 and saturated humidity.

Foxp3 Primers synthesized by Shanghai Shenggong Bioengineering Service Co., Ltd.;

Foxp3 primers were synthesized by Sangon Biotech (Shanghai) Co. Ltd:

Primer sequence P1:5'-TCCGATTACCGGCGCATCACG,

Primer sequence: P1: 5'-TCCGATTACCGGCGCATCACG-3',

P2 : 5'-CTCCAGCAGCTCGAAAAGGCA 。

P2: 5'-CTCCAGCAGCTCGAAAAGGCA-3'.

Main Experimental Instruments and Apparatuses

W-CJ-IF bechtop: Suzhou Purification Equipment Co. Ltd; carbon dioxide incubators: Heal Force HF240; low-temperature and high-speed tabletop centrifuge (Sigma): B. Braun Biotech International GmbH (BBI); a flow cytometry (EPICS, type X): Beckman Coulter Inc. (U.S.A); a ultraviolet microspectrophotometer: Amersham Pharmacia Biotech Co. Ltd (U.S.A); a PCR amplification instrument (PERKIN ELMER-2231): PerkinElmer; KB2117-II multi-function electrophoresis apparatus: Shanghai Sibas Biotechnology Development Co. Ltd; Gel-Pro IMAGER The Integrated solution (Gel Document System) 60-2517: Med ia Cybernetics Inc., U.S.A.

Isolation of CD4+T cells

Preparation of Spleen Mononuclear Cell Suspensions

After C57BL/6 mice were sacrificed, the spleens were obtained under sterile conditions and added 20ml of RPMI1640 solution (containing 2% fetal bovine serum) 20 ml.The mixture was placed on the aseptic 100-mesh steel milling net, milled repeatedly, filtered for 2 times,

centrifugated at 1000 rpm for 10 minutes, dicarded the supernatant.In the remaining mixture, add Tris-NH4CL buffer breaking red blood cells, set aside 5 minutes, centrifugate at 1000 rpm for 5 minutes, phosphate buffer saline (PBS) was resuspended, centrifugated at 1000 rpm for 5 minutes , dicarded the supernatant. The concentration of cells was regulated as 10^7 /ml.

CD4+T Cells Sorted by Magnetic Beads

1×10^7 cells were taken out and resuspended with 2 mol/L EDTA sorting buffer containing 5 g/L BSA, mixed with appropriate amount of biotin-antibody cocktail for 10 minutes of incubation under 4℃, and mixed again with anti-biotin micro beads for 15 minutes of incubation under 4℃, which were centrifugated with 1000 rpm for 10 minutes after washing with buffer, discarded the supernatant;The precipitation was resuspended with 500ul buffer; then, the LD separation column was put in the MidiMACS separator and washed with 1 ml buffer for 2 times; the cell suspension was added into the separation column and the cells flowed out from the sorting column were obtained to be CD4+ T cells.

CD4+CD25+T Cells Sorted by Magnetic Beads

Prepared spleen mononuclear cell suspension was resuspended with 2 mol/L EDTA buffer containing 5 g/L BSA, mixed with appropriate amount of biotin-antibody cocktail for 10 minutes of incubation under 4℃, and mixed again with anti-biotin microbeads for 15 minutes of incubation under 4℃, centrifugated at 1000 rpm for 10 minutes after washing with buffer, removed supernatant ; 500 μl buffer was employed for precipitation and resuspension; the LD separation column was set in the MidiMACS separator and washed with 1 ml buffer for 2 times.The cell suspension was added into the separation column .The negative selection was obtained to be CD4+T cells, which was centrifugated at 1000 rpm for 5 minutes, discarded the supernatant , washed with PBS for 1 time, added with 10 μg anti-CD25 antibodies for 30 minutes of incubation under 4℃, centrifugated with 300×g for 10 minutes, removed the supernatant, and re-washed for 1 time to obtain pure CD4+CD25+T cells (It was determined by a flow cytometry that the purity of the sorted CD4+CD25+ cell population was more than 95%).

Experiment of CD4+T-Cell Proliferation and its Treatment with Rapamycin

The proliferation level of spleen CD4+T cells was detected by ^{3}H-TdR incorporation method. The pure CD4+T cells obtained above were used as responding cells, prepared with RPMI-1640 culture medium as 2×10^6/ml; 100 ul was got and added into a 96-well culture plate to conduct the following experiment, involving the Group A (control group) as pure responding cells (R), Group B as R + Cyclosporin A (10 nM) group and Group C as R+ Rapamycin (20 ng/ml) group; wells were transfected in triplicate for each group. Each group was stimulated with anti-mouse αCD3 monoclonal antibodies (5 μg/ml), and incubated in the 37℃ and 5%CO2 incubator for 3 days. At the last 16 hours from the ending time, 1 μCi^{3}H-TdR was added into each well. After the culturing, the cultures were collected on the glass fiber filter by a multi-head cell collecting device (produced by Shanghai Yuejin Medical Instruments Co. Ltd), fixed with 100 g/L trichloroac acid, dried under 60℃ after washing with 95% (volume fraction) ethanol and put in a scintillation vial with scintillation fluid; the ^{3}H-TdR incorporation value of cells to be measured was measured by a liquid scintillation counter (Wallac).The result was expressed as a mean CPM (counts per minute) ±SD (standard deviation) (pulse count / minute).

Expression Level of TGF-β of CD4+CD25+T Cells Detected by Semiquantitative RT-PCR

1×10^6 cells were taken out and added 1.0 ml TRIzol, thoroughly homogenized and repeatedly beat, mixed upside down to equality invertedly, and set aside on the ice for 5 minutes, added 200 ul chloroform, shook invertedly for 15 seconds, centrifugated at 12000 rpm under 4℃ for 15 minutes after a quiet rest of 15 minutes on the ice; upper colorless liquid phase (RNA phase) was put in a new eppendorf

tube and added with equal volume of propanol, invertedly mix, centrifugated at 13000 rpm under 4℃ for 10 minutes after a quiet rest of 10 minutes on the ice, removed supernatant; 1ml, 4℃, 75% ethanol (prepared by DEPC) was added in above samples to invertedly mix and be centrifugated at 10000 rpm under 4℃ for 5 minutes; vacuum drying was performed for 30 minutes without supernatant. 20 ul DEPC was used to dissolve and mix RNA fully. 2 ul total prepared RNA sample was taken out to measure the optical density (OD) value of UVA at wavelengths of 260 nm and 280 nm by an ultraviolet spectrophotometer, and to calculate the ratio of OD260/OD280, so as to identify the purity of mRNA. PCR products were placed in 1.2% agarose gel for 1 hour of electrophoresis at 80V voltage,observed under an ultraviolet transilluminator and analyzed with Gel-Pro IMAGER 60-2517 image analysis system to detect the OD value of each band and calculate the ratio of the OD value between TGF-β and β-action, i.e. the relative expression amount of targeted RNA; computational formula: relative amount = the OD value of the product electrophoretic band / the OD value of β- action product electrophoretic band

Proliferation of CD4+CD25+T Cells, Expression of Foxp3 and the Treatment with Rapamycin

The purified CD4+CD25 T cells sorted by magnetic beads on step 1.4.3 were used to prepare as 2×10^6/ml with RPMI-1640 culture medium; 100 ul was added into a 96-well plate to conduct the following experiment, involving the Group A (control group) as pure responding cells (R), Group B as R + Cyclosporin A (10 nM) group, and Group C as R+ Rapamycin (20 mg/ml) group; wells were transfected in triplicate for each group. Each group was stimulated with IL-2 (100 U/ml), anti-mouse αCD3 monoclonal antibodies (5μg/ml) and αCD28 monoclonal antibodies（2μg/ml) and were incubated in a 37℃, 5%CO2 incubator for 7 days; then, a flow cytometry was used to detect the proportion of CD4+CD25+T cells and the change of Foxp3+ phenotype.

Inhibition Tests of CD4+CD25+T Cells

C57BL/6 mouse spleen CD4+CD25+T cells and CD4+CD25-T cells were sorted with magnetic beads as the above method. C57BL/6 mouse CD4+CD25-T cells (2×10^6/ml) were used as responding T cells. The splenocytes of BALB/c mice spleen were regarded as stimulator cells （S）after inactivating with Mitomycin C (30 μg/ml) under 37℃ for 30minutes. The responding cells and stimulator cells were mixed on the proportion of 10:1 and added in a 96-well plate containing 10% FBS culture medium; CD4+CD25+T cells and responding cells were mixed in the MLR system on different proportions (1:1, 1:2, 1:4); wells were transfected in triplicate for each group; responding cells and CD4+CD25+T cells were incubated in a 37℃, 5%CO2 incubator for 3 days as controls. At the last 16 hours from the ending time, 1μCi ^{3}H-TdR was added into each well. After the culturing, the cultures were collected on the glass fiber filter by a multi-head cell collecting device (produced by Shanghai Yuejin Medical Instruments Co. Ltd）, fixed with 100 g/L trichloroacetic acid, dried under 60℃ after washing with 95% (volume fraction) ethanol and put in a scintillation vial with scintillation fluid; the ^{3}H-TdR incorporation value of cells to be measured was measured by a liquid scintillation counter (Wallac). The result was expressed as a mean CPM (counts per minute) ± SD (standard deviation) (pulse count / minute).

Activation and Expression of SMAD2/3 and Foxp3 by Western Blotting

The purified CD4+CD25+T cells were sorted with magnetic beads as the above method to treat on the following groups: the Group A (control group) as pure responding cells (R), Group B as R + anti-TGF-β (2ng/ml) group, Group B as R + Cyclosporin A (10 nM) group, and Group C as R + Rapamycin (20 mg/ml); wells were transfected in triplicate for each group. Each group was added with IL-2 (100 U/ml), anti-mouse αCD3 monoclonal antibodies (5 μg/ml), and αCD28 monoclonal antibodies (2 μg/ml) in a 37℃ and 5%CO2 incubator for co-culture. After 3 days , the cells were collected and added to RIPA buffer at 3ml/g, added phenylmethylsulfonyl

fluoride, beaten directly with a 1 ml suction nozzle,and placed on the ice bath for 30 minutes; the samples were transferred into a 1.5 ml centrifuge tube and centrifugated at 12000 rpm at speed under 4℃ for 30 minutes at high speed; the supernatant was transferred carefully into a clean and aseptic centrifuge tube for storage under -20℃. A small amount of supernatant was taken out to conduct protein quantitation according to the instruction sheet of the test kit for protein concentration with bicinchoninic acid (BCA) method from KeyGEN Company; RIPA was used as the blank control. Each experiment was repeated for 4 times; and representative electrophoresis results of Western blot were selected, scanned into a computer and treated by image analysis software Gel-Pro Analyzer (Ver 3.0).

Statistical analysis

The experimental data were expressed as mean ± standard deviation (M±SD). SSPS12.0 was used for statistical treatment of all data. One-Way ANOVA was used for analysis. The level of inspection showed $\alpha = 0.05$ and $P < 0.05$, suggesting significant difference.

3. RESULTS

Rapamycin Promoted the In Vitro Cultured CD4+T Cells to Produce TGF-β

TGF-β is a group of newly discovered TGF-β superfamily that could regulate cell growth and differentiation. At first, the biological function of TGF-β was mainly studied in inflammation, tissue repair and embryonic development etc. It has been revealed in recent years that TGF-β plays an important regulating role in the growth, differentiation and immune function of cells. The functions of TGF-β1, β2 and β3 are similar; generally, TGF-β could stimulate cells originated from mesenchyme and inhibit cells from the epithelium or neural ectoderm. TGF-β inhibited the proliferation of immunologically competent cells: ① Inhibit the mouse hemopoietic precursor cells induced by IL-3, GM-CSF and M-CSF and the colony formation of LTBMC , and reduce the reactivity of megakaryocytes to IL-3T and CSF. ② Inhibit the proliferation of thymocytes induced by ConA or ConA combined with IL-2 and IL-6. ③Inhibit proliferation of T cells stimulated by mitogen and allogenic antigen or IL-2-dependent T cell growth. ④ Inhibit the proliferation of IL-2-dependent B cells after being stimulated by SAC. More and more studies have confirmed that TGF-β signal is critical in the activation of Tregs function. The article by Ingrid et al. found that Rapamycin could stimulate the production of TGF-β, but it is not clear whether the effect of Rapamycin on Tregs is related to the induction of TGF-β. In the in vitro experiment, we first observed the effect of Rapamycin on the secretion of TGF-β in CD4+T cells and its proliferation ability.CD4+T cells of High-purity C57BL/6 mouse spleen were cultured in a 96-well plate (2×10^6/ml) with RPMI-1640 medium containing 10% fetal bovine serum and conduct as the following groups: the Group A (control group) as pure responding cells (R), Group B R+ Cyclosporin A (10 nM) group, and Group C as R+Rapamycin (20 ng/ml) group; wells were transfected in triplicate for each group. Each group was stimulated with anti-mouse αCD3 monoclonal antibodies (5 μg/ml), and incubated in the 37℃ and 5% CO2 incubator for 3 days. The level of TGF-β of the supernatant was assayed by the enzyme-linked immunosorbent assay (ELISA) kit. The results showed that compared with the control group, the level of TGF-β secreted by Rapamycin-treated CD4+T cells increased by nearly 2.5 times, suggesting significant differences ($P<0.01$); while the level of TGF-β secreted by Cyclosporin A-treated CD4+T cells reduced, demonstrating no significant difference compared with that in the control group ($P>0.05$) (as shown in the Fig. 1A). It was suggested that Rapamycin could promote CD4+T cells to secrete TG-β, but there is no effect of Cyclosporine A. The proliferation of CD4+T cells were assayed with ^{3}H-TdR incorporation method, which indicated that compared with that in the control group, Rapamycin and Cyclosporin A both can inhibited significantly the proliferation of T cells stimulated by αCD3 monoclonal

antibodies (P<0.01); while no significant differences were noted in the inhibitory effect between Rapamycin and Cyclosporin A (P>0.05) (as shown in the Fig. 1B), suggesting that Rapamycin and Cyclosporin A could play a role in the anti-transplant rejection by inhibit the proliferation of CD4+T cell effectively and directly, but only Rapamycin could promote CD4+T cell to secrete TGF-β.

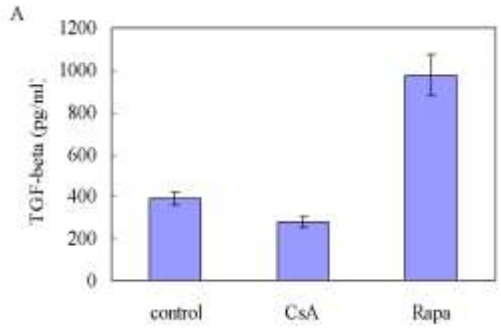

Fig. 1A 2.1 Rapamycin Promote CD4+T Cells Cultured in Vitro to Produce TGF-β

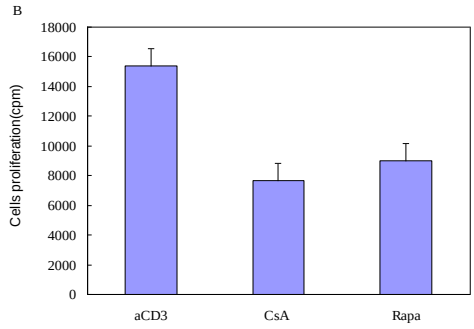

Fig. 1B Effects of Different Drugs on the Proliferation of Spleen CD4+T Cells

Effects of Rapamycin on the Differentiation of CD4+T Cells into CD4+CD25+T Cells

Effects of Rapamycin on the differentiation of CD4+T cells into CD4+CD25+T cells were further analyzed. The purified CD4+T cells were collected and prepared with RPMI-1640 medium as 2×10^6/ml, 100ul was added into a 96-well culture plate to treat as following groups: the Group A (control group) as pure responding cells (R), Group B as R+Cyclosporin A (10 nM) group, and Group C as R + Rapamycin (20 ng/ml) group; wells were transfected in triplicate for each group. Each group was added with IL-2 (100 U/ml), anti-mouse αCD3 monoclonal antibodies (5μg/ml) and αCD28 monoclonal antibodies (2 μg/ml) to incubate in a 37℃ and 5%CO2 incubator for 5 days; then, the proportion changes of CD4+CD25+Tregs were detected with a flow cytometry,which suggested that the proportion of CD4+CD25+Tregs in the Rapamycin group accounted for 65.21% in CD4+T cells, and reduced slightly in comparison of that in the negative control group (69.22%); there was no statistically significant difference (P>0.05). In the Cyclosporin A group, the proportion of CD4+CD25+Tregs accounted for 41.25% in CD4+T cells, and obviously decreased compared with that in the negative control group (69.22%); statistically significant differences were noticed (P<0.01) (as shown in the Fig. 2A).

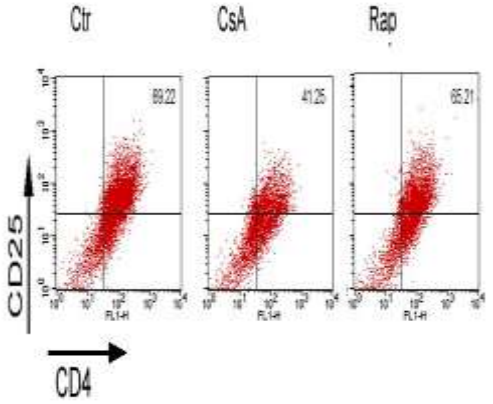

Fig. 2A Proportion Changes of CD25+T Cells with Different Treatment for CD4+T Cells

Effects of Rapamycin on the Proliferation of CD4+CD25+T Cells and Expression of Foxp3

The effect of Rapamycin on the expression of Foxp3 was further analyzed in CD4+CD25+T cells. Purified CD4+CD25+T cells were collected to prepare as 2×10^6/ml with RPMI-1640 medium; 100ul was taken out and added into a 96-well culture plate to treat on the following groups: the Group A (control group) as pure responding cells (R), Group B as R+ Cyclosporin A (10 nM) group, and Group C as R + Rapamycin (20 ng/ml) group; wells were transfected in triplicate for each group. Each group was stimulated with IL-2 (100 U/ml), anti-mouse αCD3 monoclonal antibodies (5μg/ml) and

αCD28 monoclonal antibodies（2μg/ml), and incubated in a 37℃, 5%CO2 incubator for 7 days; a flow cytometry was used to detect the change of Foxp3. The results showed that the proportion of FoxP3+T cells in the Rapamycin group increased to 53.7% (P<0.05) than that in the control group (40.2%); while FoxP3+T cells in the Cyclosporine A group were inhibited obviously to 23.6% (P<0.01) (as shown in the Fig. 2B). As was suggested, Rapamycin could promote the expression of FoxP3 in CD4+CD25+T cells cultured in vitro, while Cyclosporine A significantly inhibited the proliferation of CD4+CD25+T cells and the expression of FoxP3.

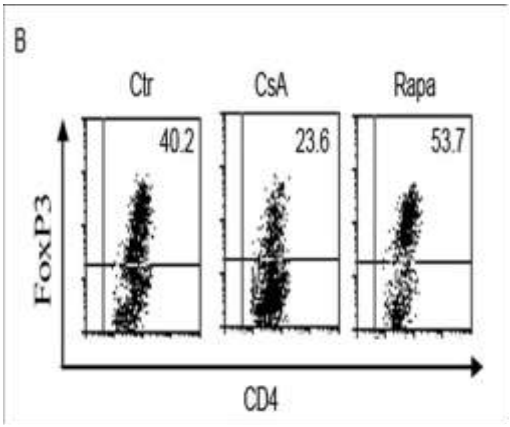

Fig. 2B Effects of Different Treatments on the Expression of Foxp3+ in Spleen CD4+CD25+Tregs

Reaction Ability of CD4+CD25+T Cells Treated by Rapamycin on the Inhibition of MLR

We investigated whether Rapamycin could promote the immune regulation of CD4+CD25+T cells. C57BL/6 mouse spleen CD4+CD25+T cells were sorted with magnetic beads; the experimental group were treated with Rapamycin as the above method. C57BL/6 mouse CD4+CD25-T cells (2×10^6/ml) were taken as responding T cells. The splenocytes of BALB/c mice spleen were regarded as stimulator cells（S）after inactivating with Mitomycin C (30 μg/ml) under 37℃ for 30minutes. The responding cells and stimulator cells were mixed on the proportion of 10:1 and added into a 96-well plate containing 10% FBS medium; Rapamycin treated CD4+CD25+T cells : Responding cells were mixed in the MLR system on the proportion of (1:1, 1:2, 1:4) ; wells were transfected in triplicate for each group, and responding cells, CD4+CD25+T cells were set as controls, which were cultured under 37℃ and 5%CO2 conditions for 3 days. ^{3}H-TdR incorporation efficiency was detected to observe the MLR response. As was shown in the Fig. 5, CD4+CD25-T cells developed almost no proliferation under no stimulation; while in the MLR system, CD4+CD25-T cells treated with stimulator cells on the proportion of 10:1 showed significant proliferation. CD4+CD25+T cells and responding cells treated with Rapamycin were mixed with MLR system on different proportion (1:1, 1:2, 1:4), which produced obvious inhibition on the proliferative responding cells stimulated, suggesting that Rapamycin could enhance markedly the inhibition of CD4+CD25+Tregs to CD4+CD25-Tregs. It was demonstrated that Rapamycin could play anti-transplant rejection by enhancing the inhibition and regulation functions of CD4+CD25+Tregs.

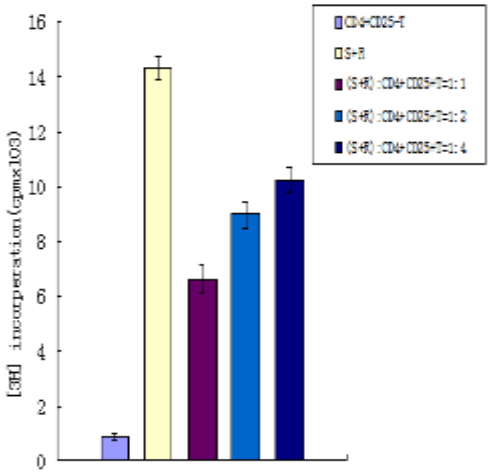

Fig. 3 Effects of Rapamycin on the Inhibitory Function of Spleen CD4+CD25+Tregs

4. DISCUSSION

Rapamycin was extracted from the hydrophil streptomyces on the Easter Island of Canada in 1975, which was regarded as a macrolide antibiotic at that time, and was revealed to have strong immunosuppressive effects in 1989. It has been confirmed that this drug can reduce

glomerulosclerosis, the vascular lesion of cardiac transplantation and the symptoms of chronic rejection [1-2]. Rapamycin is similar with immunosuppressive agent FK506 and Cyclosporin A on the structure, and plays its effects by combining intracellular immunophilin FK506 binding-protein 12 (FKBP12). However, Rapamycin cannot inhibit the activity of T cell receptor (TCR)-induced calcineurin, which is different from FK506 and Cyclosporin A.Furthermore, Rapamycin-FKBP12 complexes can inhibit serine / threonine protein kinase (called the mammalian target of Rapamycin); protein synthesis and cell cycle progression need to activate the protein kinase [3]. Hence, Rapamycin blocks the acknowledge signal of cytokines / growth factors, and FK506 and Cyclosporine A can exert their inhibitory effects by blocking T cell receptor-induced activation [2-6].Therefore, Rapamycin can prevent the T cell division cycle from G1 phase to S phase; promote T cell receptor to induce non-reactivity of T cells (even with co-stimulation); allow inducing operational tolerance. In addition, the combination of Rapamycin and FKBP12 can remove the blocking effect of FKBP12 to TGF-β mediated signaling events, suggesting that Rapamycin may act on the TGF-β signaling pathway [7-12].

TGF-β, a multifunctional cytokine that can influence a variety of cell types, can mediate apoptosis and stimulate fibroblast growth and the following extracellular matrix formation [13-16]. In the immune system, it can exert important immunoregulatory and anti-inflammatory functions, involving the inhibition of the proliferation of T cells and B cells [17-20], inactivate macrophages and promote immune deviation from TH1 to TH2 [21-25]. In the specific model, exogenous TGF-β can delay the allograft rejection and reduce the severity of autoimmune diseases. Additionally, mutations of TGF-β coding gene in mice cause severe multi-organ inflammation and early death, indicating the important role of this cytokine in the basic process of immunoregulation [26-28].

The study of Yongzhong Liu et al. has demonstrated that TGF-β signal is indispensable to the development of natural CD4+CD25+Foxp3+Tregs in thymus gland, and can result in fibrosis of transplanted organs or tissues; during the early allograft rejection, the increasing expression level of TGF-β may benefit the inhibition of acute immune responses; however, the sustained expression of TGF-β may increase fibrosis of transplanted organs or tissues, and cause chronic rejection. Rapamycin can inhibit the fibrosis of transplanted organs or tissues, which has been reported in literature. TGF-β secreted by cell just a short time ago is in the inactive state. The combination of Rapamycin and FKBP12 can abolish the inhibitory effect of FKBP12 on TGF-βRI activity, promote heterodimers complexes combination formed by TGF-β, TGF-βRIand TGF-βR IIsubunits and activate the kinase domain in recipient cells, resulting in the phosphorylation and activation of Smad family members, and then regulating TGF-β-dependent gene transcription and expression [29-30]. Hence, Rapamycin can promote the activation of TGF-β.

Increasing studies have confirmed that TGF-β is critical in the differentiation and proliferation of CD4+CD25+Tregs, a class of T cells with immunosuppressive action, whose functions are different from T cell subsets with immunomodulatory functions in Th1 and Th2, and involve actively in the regulation and control of immune response and immune tolerance, to play an important regulation and control role in the regulation of autoimmune tolerance, tumor immunity and transplantation immunity. Although CD4+CD25+T cell has an extremely important role in the induction and maintenance of immunological tolerance of transplantation, it remains unknown about effects of immunosuppressive agents widely used for treating organ-graft refection on CD4+CD25+T cells. The experiment suggested that different immunosuppressive agents had different effects on CD4+CD25+T cells; some immunosuppressive agents inhibit rejection, but also block the activation and establishment of immune tolerance. A latest study has demonstrated that

Rapamycin and other immunosuppressive agents can promote in vitro amplification effects of CD4+CD25+T cells. However, little is known about the molecular mechanism by which Rapamycin promotes the production of Tregs [31-32].

In this experiment, we analyzed whether Rapamycin promoted the proliferation of CD4+CD25+Foxp3+Tregs with TGF-β to exert an immune regulation role, researched the action and molecular mechanism by which Rapamycin promoted the production of Tregs, and investigated that immunosuppressive agents could induce the proliferation of specific CD4+CD25+Tregs and enhance the immunosuppressive ability to effector cells when organs were transplanted, in order to benefit the establishment of immunological tolerance of transplantation and long-term survival of organ grafts.

The experiment result has revealed that effects of Rapamycin to CD4+CD25+Tregs are related to the induction of TGF-β. In addition, we also have discovered that Rapamycin can enhance the ability of TGF-β to induce the production of CD4+CD25+Tregs.

High-purity C57BL/6 mouse spleen CD4+T cells were treated with different in vitro culture systems [A: the control group; B: Cyclosporin A (10 nM); C: Rapamycin (20 ng/ml)] and detected with different methods, suggesting that compared with that in the control group, Rapamycin could enhance significantly the level of TGF-β secreted by CD4+T cells; while the level of TGF-β secreted by Cyclosporin A-treated CD4+T cells reduced. The proliferation of CD4+T cells was assayed with the ^{3}H-TdR incorporation method, which revealed that Rapamycin and Cyclosporin A could inhibit significantly the proliferation of T cells stimulated by αCD3 monoclonal antibodies; whereas no significant differences were noted in the inhibitory effect between Rapamycin and Cyclosporine A. Effects of Rapamycin on the proportion of CD4+CD25+Tregs and expression of FoxP3+ were analyzed, which showed that the expression of FoxP3+ in the Rapamycin group increased markedly compared with that in the negative control group, while the proportion of CD4+CD25+Tregs and expression of FoxP3+ in the Cyclosporin A group reduced significantly than that in the negative control group. Effects of Rapamycin on the inhibitory function of CD4+CD25+Tregs were further observed, discovering that in the MLR reaction, in comparison of that in the negative control group (the Tregs group), Rapamycin could enhance significantly the inhibitory function of CD4+CD25+Tregs to CD4+CD25-T cells [31-34].

In conclusion, Rapamycin can significantly increase the expression of CD4+CD25+regulatory Foxp3 in mice in vitro, and also maintain the function of these cells at the same time. It is demonstrated that Rapamycin can promote the proliferation of CD4+CD25+T cell subsets cultured in vitro, and the expression of their FoxP3+; while Cyclosporine A significantly inhibits the proliferation of CD4+CD25+T cell subsets and the expression of FoxP3+. Rapamycin can promote the secretion of TGF-β, and trigger Smad-2/3 phosphorylation to activate TGF-β signal transduction pathways, in order to promote the proliferation of CD4+CD25+Tregs subsets and the expression of FoxP3+, as reveals that Rapamycin can involve the cellular mechanism of the anti graft rejection by promoting the function of CD4+CD25+Tregs.

5. COMPETING INTERESTS

This work was performed in collaboration with Guangdong Cardiovascular Institute. Ming-Jie Mai is the archiater of Guangdong Cardiovascular Institute. The authors declare that they have no conflicts of interest.

6. ACKNOWLEDGMENTS

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