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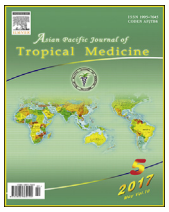

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Preoperative application of combination of portal venous injection of donor spleen cells and intraperitoneal injection of rapamycin prolongs the survival of cardiac allografts in mice

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ABSTRACT

Objective: To investigate the effects of preoperative portal venous injection of donor spleen cells (PVIDSC) and intraperitoneal injection of rapamycin in the acute rejection of cardiac allograft in mice and the underlying mechanisms.

Methods: Homogenous female B6 mice and BALB/c mice were used as recipients and donors of heart transplantation. These mice were randomly divided into different groups and received PVIDSC alone, rapamycin alone, or PVIDSC and rapamycin combined therapy. In addition, the underlying mechanism was studied by measuring a number of cytokines.

Results: Preoperative combination of PVIDSC and intraperitoneal injection of rapamycin significantly prolonged the survival of heterotopic cardiac allograft in mice, but had no effects on the survival time of cardiac allografts in mice pre-sensitized by skin grafting. Preoperative combination of PVIDSC and intraperitoneal injection of rapamycin increased the expression of IL-10 and Foxp3 and reduced the expression of INF-γ. Short-term preoperative administration of rapamycin promotes the expression of $CD4^+CD25^+Foxp3^+$ regulator T cells. However, preoperative using alone of rapamycin, or combination of PVIDSC and rapamycin had no effects on the inhibition of proliferation of memory T cells.

Conclusions: Preoperative application of combination of PVIDSC and rapamycin significantly prolonged the survival time of cardiac allografts in mice but not in mice pre-sensitized by skin grafting. This may be explained by the fact that combination of PVIDSC and rapamycin inhibited the cellular immune response and induced the expression of IL-10 from Tr1 cells and $CD4^+CD25^+FoxP3^+$ regulatory T cells.

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1. Introduction

In the 1950s, Dr. Joseph Murray conducted kidney transplant between a pair of twin brothers, which represents a milestone of the human organ transplant [1]. For over 50 years, great success has been achieved in the field of organ transplantation including successful transplantation of kidney, liver, and heart. Organ transplantation has become the most effective treatments to save lives of patients with organ failure. The application of immunosuppressants has greatly increased the success rate of organ transplantation and improved the survival time of transplanted organs. However, long-term use of large dose of immunosuppressants increases the chance of opportunistic infections and tumors, which seriously affected the long-term effects of organ transplant and quality of life of recipients [2]. Therefore, identification and application of immunosuppressants of low toxicity are highlighted in the field of organ transplantation.

Rapamycin is a novel macrolide immunosuppressant. By binding to different cytokine receptors, rapamycin inactivates signal transduction to arrest T cells and other cells at the G1 phase. In this study, rapamycin was selected to study its immunosuppressive effects in cardiac transplantation because it inhibits the repulsion against spleen cells from donors and inhibits graft-versus-host disease (GVHD). In addition, rapamycin can also promote the production of regulator T cells [3], which thereby induces immune tolerance.

In recent years, it has been reported that spleen cells play an important role in the induction of immune tolerance [4]. Studies demonstrated that importing donor spleen cells induced donor-specific immune tolerance and prolonged graft survival time [5,6]. Injection of donor spleen cells via portal venous may be more efficient than via peripheral vein to induce immune tolerance. In this study, we evaluated the effects of preoperative application of PVIDSC and intraperitoneal injection of rapamycin in the acute rejection of cardiac allograft in mice and skin grafts pre-sensitized mice, and to find an efficient way inducing immune tolerance using small amount of immunosuppressant.

2. Materials and methods

2.1. Animals

Homogenous female B6 (H-2^b) mice and BALB/c (H-2^d) mice were used as recipients and donors of heart transplantation, respectively, in this study. All mice (8–12 weeks old and 19–22 g) were purchased from the Shanghai SLAC Laboratory Animal Co., Ltd., Chinese Academy of Sciences.

Twenty-four female BALB/c mice were randomly divided into four corresponding groups (D1, D2, D3, and D4) as heart donors. Twenty-four female B6 mice were randomly divided into four groups (R1, R2, R3, and R4) as recipients. Twenty-four female B6 mice pre-sensitized by skin grafting were randomly divided into four groups (Rsg1, Rsg2, Rsg3, and Rsg4) as recipients and used as control. No preoperative treatments were given to the R1 and Rsg1 group animals. The R2 and Rsg2 group animals received PVIDSC on the 7th day prior to heart transplantation. The R3 and Rsg3 group animals received intraperitoneal injection of rapamycin (1.5 mg/kg/d) for seven days (from the 7th day prior to heart transplantation to the operation day). The R4 and Rsg4 group animals received a combinative treatment of PVIDSC and rapamycin as mentioned above.

Four to six weeks prior to heart transplant, B6 mice in the Rsg1, Rsg2, Rsg3, and Rsg4 groups received skin graft of full-thickness of back skin from BALB/c donor mice [7]. The skin graft is circular with a diameter of about 1.0 cm.

2.2. Preoperative treatments

The spleens of BALB/c donor mice were removed and lysed using red blood cell lysates to produce single cell suspension. The spleen cells from donor animal were injected to B6 recipient mice (0.3 mL; 1×10^7 cells/animal) on the 7th day prior heart transplant via portal vein. Intraperitoneal injection of rapamycin for seven days (from the 7th day prior to heart transplantation to the operation day) was conducted.

Rapamycin (BBI Co., Canada) was dissolved in ethanol and diluted with sodium carboxymethylcellulose and stored at 4 °C. Rapamycin was intraperitoneally injected to B6 recipient mice (1.5 mg/kg).

2.3. Cervical heterotopic heart transplantation

BALB/c mice were used as donors and B6 mice were used as recipients. Cervical heterotopic heart transplantation was conducted according to previous report [8]. Cardiac allograft was observed daily for pulse to evaluate its function. Exclusion was defined as no pulse is observed.

2.4. Histopathological examination of cardiac allografts

The bottom of cardiac allografts was dissected on the 7th day after heart transplantation for histopathological examination. The autopsy was fixed with 10% formaldehyde and embedded paraffin for hematoxylin and eosin staining and examined under light microscopy. Organ rejection grading was evaluated according to the ISHLT reference standard [9,10] and compared between groups.

2.5. Flow cytometry

Monoclonal antibodies include PE-cy5-CD4 (clone GK1.5), PE-cy5-CD8 (clone 53–6.7), FITC-CD44 (clone IM7) and controls were purchased from Biolegend Corporation (USA). BALB/c donor mice were sacrificed by cervical disarticulation and spleens were harvested. Single cell suspensions were prepared by grinding the tissues with the plunger of a 5 mL disposable syringe and were then suspended in RPMI1640 medium. Splenocytes were treated with a hemolysis buffer (17 mM Tris–HCl and 140 mM NH_4Cl, pH 7.2) to remove RBCs [11]. Erythrocytes were lysised using erythrocyte lysate and the suspension went through 400 mesh strainer. The cells were then washed twice using PBS. After adjusting cell number and volume, fluorescence monoclonal antibodies were added and incubated in dark at room temperature for 30 min. Then, cells were washed twice before flow cytometry assay. Flow cytometry was conducted in Beckman EpicsXL (Beckman, USA). The flow cytometry data were analyzed using the FlowJo software.

2.6. Quantitative real-time RT-PCR (qRT-PCR)

Total RNA was isolated from the apical portion of the cardiac allografts using Trizol RNA isolation kid (Invitrogen, USA). The StepOne Real-Time PCR kit (QPK-212, TOYOBO, Japan) was used to do reverse transcription and PCR amplification. Reaction step: 95 °C denaturation 2 min; 95 °C 30 s, 55 °C 30 s, 72 °C 30 s 40 cycles. Detection of amplification results was conducted on the ABI stepone system (Biosystems). β-actin was used as a internal control to evaluate the expression levels of IFN-γ, IL-2, IL-10, and Foxp3 in cardiac allografts. Triplicates were used for each sample. PCR primer used for qRT-PCR were as follows:

β-actin: forward 5′-CATCCGTAAA-GACCTCTATGCCAAC-3′, and reverse 5′-ATGGAGC-CACCGATCCACA-3′; IFN-γ: forward 5′CGGCACAGTCATTGAAAGCCTA-3′, and reverse 5′-GTTGCTGATGGCCTGATTGTC-3′; IL-2: forward 5′-GGAGCAGCTGTTGATGGACCTAC-3′, and reverse 5′-AATCCAGAACATGCCGCAGAG-3′; IL-10: forward 5′-GACCAGCTGGACAACATACTGCTAA-3′, and reverse 5′GATAAGGCTTGGCAACCCAAGTAA-3; Foxp3: forward 5′- CAGCTCTGCTGGCGAAAGTG -3′, and reverse 5′-TCG.TCTGAAGGCAGAGTCAGGA-3′.

2.7. Statistical analyses

Survival analysis was conducted using the Kaplan–Meier method. *T*-test was used to compare the results of graft rejection score, flow cytometry, and qRT-PCR between different groups. $P < 0.01$ was considered as significantly statistical difference.

3. Results

3.1. Preoperative combination of PVIDSC and rapamycin significantly prolonged the survival of cardiac allograft in mice without skin grafting

The survival time of cardiac allograft of each group (R1, R2, R3, and R4) was shown in Figure 1. The R4 group (combination of PVIDSC and intraperitoneal injection of rapamycin) had the longest survival time of cardiac allograft among these four groups, with an average survival time of 36.0 days, and a maximum survival time of 57.0 days ($P < 0.05$). The average survival time of cardiac allograft in groups R1, R2 and R3, were 7.3, 10.6, and 9.8 days, respectively (Figure 1).

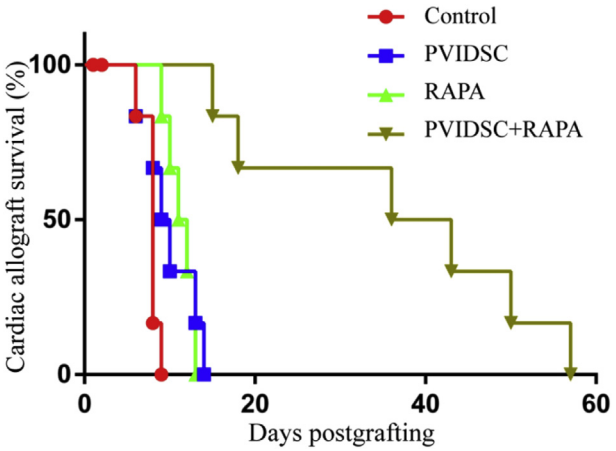

Figure 1. Comparison of the survival time of allogeneic cardiac grafts in mice without skin grafting (6 animals in each group).
The control group (R1): No preoperative treatments. The R2 group: animals received PVIDSC (0.3 mL, 1×10^7 spleen cells/animal) on the 7th day prior to heart transplantation. The R3 group: animals received intraperitoneal injection of rapamycin (1.5 mg/kg/d) for seven days (from the 7th day prior to heart transplantation to the operation day). The R4 group: animals received a combinative treatment of PVIDSC (0.3 mL, 1×10^7 spleen cells/animal) on the 7th day prior to heart transplantation and intraperitoneal injection of rapamycin (1.5 mg/kg/d) for seven days (from the 7th day prior to heart transplantation to the operation day).

3.2. PVIDSC combined with rapamycin inhibited the expression of IFN-γ and IL-2, and promoted the expression IL-10 and Foxp3 in mice

Cardiac allografts were removed on the 7th day after heart transplantation. Total RNA was isolated from the apical part of cardiac allografts and the expression of IFN-γ, IL-2, IL-10, and *Foxp3* genes were examined using qRT-PCR. Compared to the control group R1, the expression of IFN-γ and IL-2 in group R4 (combination of PVIDSC and rapamycin) significantly reduced ($P < 0.001$), but the expression of IL-10 and *Foxp3* significantly increased ($P < 0.001$) (Figure 2). Therefore, it was concluded that preoperative application of combination of PVIDSC and rapamycin significantly inhibited the production of inflammatory cytokines and promoted the proliferation of $CD4^+CD25^+Foxp3^+$ regulatory T cells and IL-10 secreting Tr1 cells. Interestingly, preoperative application of PVIDSC alone reduced the expression of IL-2, IL-10, and *Foxp3* ($P < 0.001$), but had no effects on the expression of *IFN-γ* ($P > 0.05$). Thus, preoperative application of PVIDSC alone didn't promote the induction of immune tolerance (Figure 2).

3.3. Preoperative short-term application of rapamycin promoted the proliferation of $CD4^+CD25^+Foxp3^+$ regulatory T cells

In vitro experiments have shown that combination of rapamycin and IL-2 promoted the induction of $CD4^+CD25^+Foxp3^+$ regulatory T cells [12]. qRT-PCR assay was used to test whether rapamycin alone has the same effects on the induction of $CD4^+CD25^+Foxp3^+$ regulatory T cells. Our results demonstrated that preoperative short-term application of rapamycin increased the expression of *Foxp3* gene ($P < 0.001$), and reduced the expression of *IFN-γ* ($P < 0.001$). However, rapamycin didn't have significant effects on the expression of IL-2 and IL-10 ($P > 0.05$). IL-2 plays an important role in the proliferation and differentiation of T cells, and the induction and proliferation of Treg. Unlike calcium blockers such as cyclosporine, rapamycin didn't inhibit the expression of IL-2 when exerting the immunosuppressive effects, which promoted the induction of $CD4^+CD25^+Foxp3^+$ regulatory T cells (Figure 2).

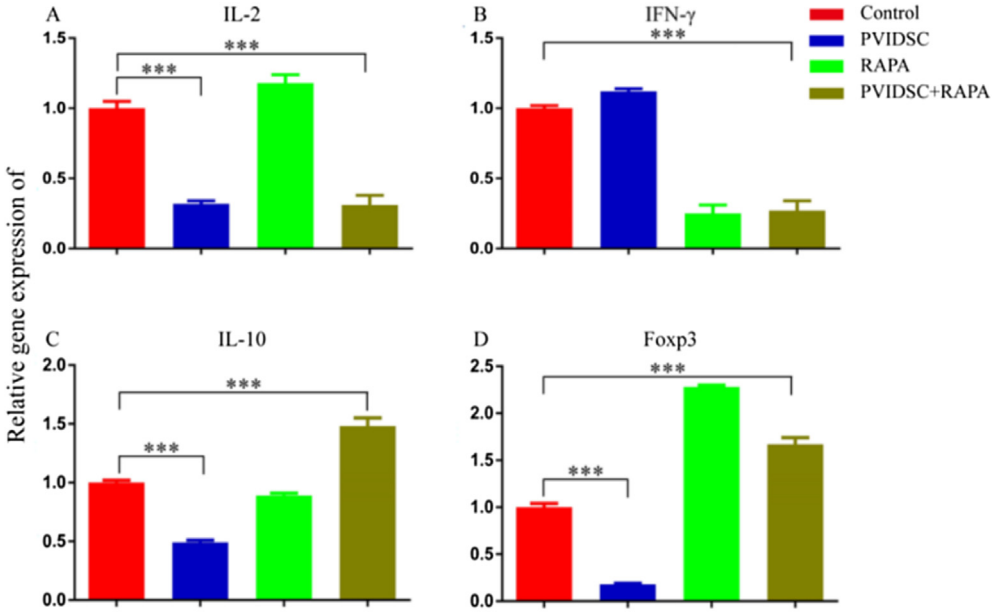

Figure 2. Comparison of the expression levels of genes *IFN-γ*, *IL-2*, *IL-10*, and *Foxp3* in allogeneic cardiac grafts in mice.
The expression levels of these four genes in allogeneic cardiac grafts were evaluated using qRT-PCR on the 7th day after heart transplantation. (A). The expression level of *IL-2*. $^{***}P < 0.001$. (B) The expression level of *IFN-γ*. $^{***}P < 0.001$. (C). The expression level of *IL-10*. $^{***}P < 0.001$. (D). The expression level of *Foxp3*. $^{***}P < 0.001$. The control group (R1): No preoperative treatments. The R2 group: animals received PVIDSC (0.3 ml, 1×10^7 spleen cells/animal) on the 7th day prior to heart transplantation. The R3 group: animals received intraperitoneal injection of rapamycin (1.5 mg/kg/d) for seven days (from the 7th day prior to heart transplantation to the operation day). The R4 group: animals received a combinative treatment of PVIDSC (0.3 mL, 1×10^7 spleen cells/animal) on the 7th day prior to heart transplantation and intraperitoneal injection of rapamycin (1.5 mg/kg/d) for seven days (from the 7th day prior to heart transplantation to the operation day).

3.4. PVIDSC and rapamycin didn't inhibit the proliferation of memory T cells

The spleen was removed from mice on the 7th day after heart transplantation and the memory T cells in spleen tissues were counted by flow cytometry. Our results demonstrated that no significant difference ($P > 0.05$) of the number of $CD4^+CD44^{high}$ and $CD8^+CD44^{high}$ T cells was observed between the control group and other groups, suggesting that combination of PVIDSC and rapamycin or alone didn't inhibit the proliferation of memory T cells (Figure 3). In addition, we observed that short-term application of rapamycin reduced the $CD4^+$ and $CD8^+$ T cells, which may be explained by the immunosuppressive capacity of rapamycin.

3.5. Combination of PVIDSC and rapamycin efficiently inhibited graft rejection

The "gold standard" of scoring cardiac allograft rejection proposed by International Society for Heart and Lung Transplantation (ISHLT) was reviewed by two reviewers [13]. The heart allograft rejection scores were grade IV, IIIB, IIIb,

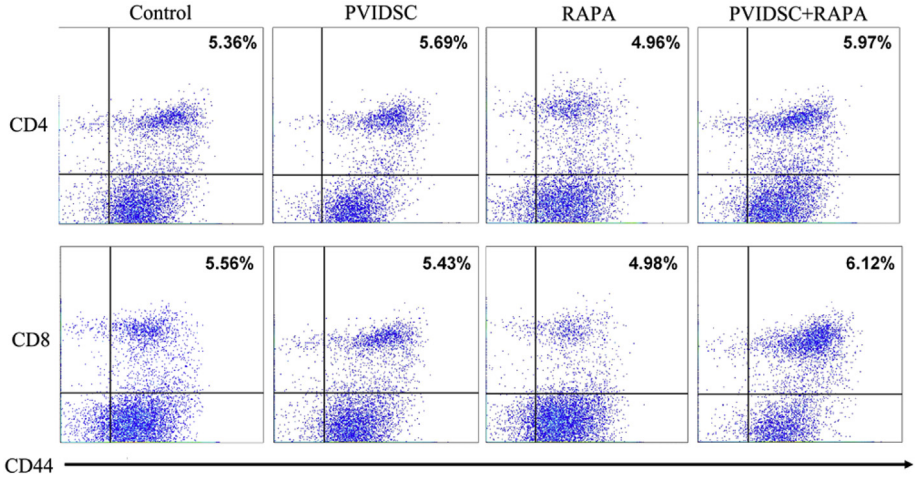

Figure 3. Combination of PVIDSC and rapamycin didn't reduce the amount of $CD44^{high}$ T cells among $CD4^+$ and $CD8^+$ T cells.
The spleen was removed from mice on the 7th day after heart transplantation and the memory T cells in spleen tissues were counted by flow cytometry using monantibodies PE-cy5-CD4, PE-cy5-CD8, FITC-CD44. Compared to the control group (R1 group), the amount of $CD44^{high}$ T cells in the other three groups (R2, R3, and R4 groups) has no significant difference.

and IA for the control group (group R1), groups R2, R3, and R4, respectively. Our results suggested that combination of PVIDSC and rapamycin efficiently inhibited the lymphocyte infiltration in cardiac allografts and protected the function of cardiac allografts. Severe graft rejection was still observed in the groups received PVIDSC or rapamycin alone (Figure 4).

3.6. Preoperative combination of PVIDSC and rapamycin had no significant influence on the survival of cardiac allograft in mice pre-sensitized with skin graft

The average survival time of cardiac allograft was 3.3 days of the Rsg1 group, 3.5 days of the Rsg2 group, 4.8 days of the Rsg3 group, and 5.2 days of Rsg4 group (Figure 5). No

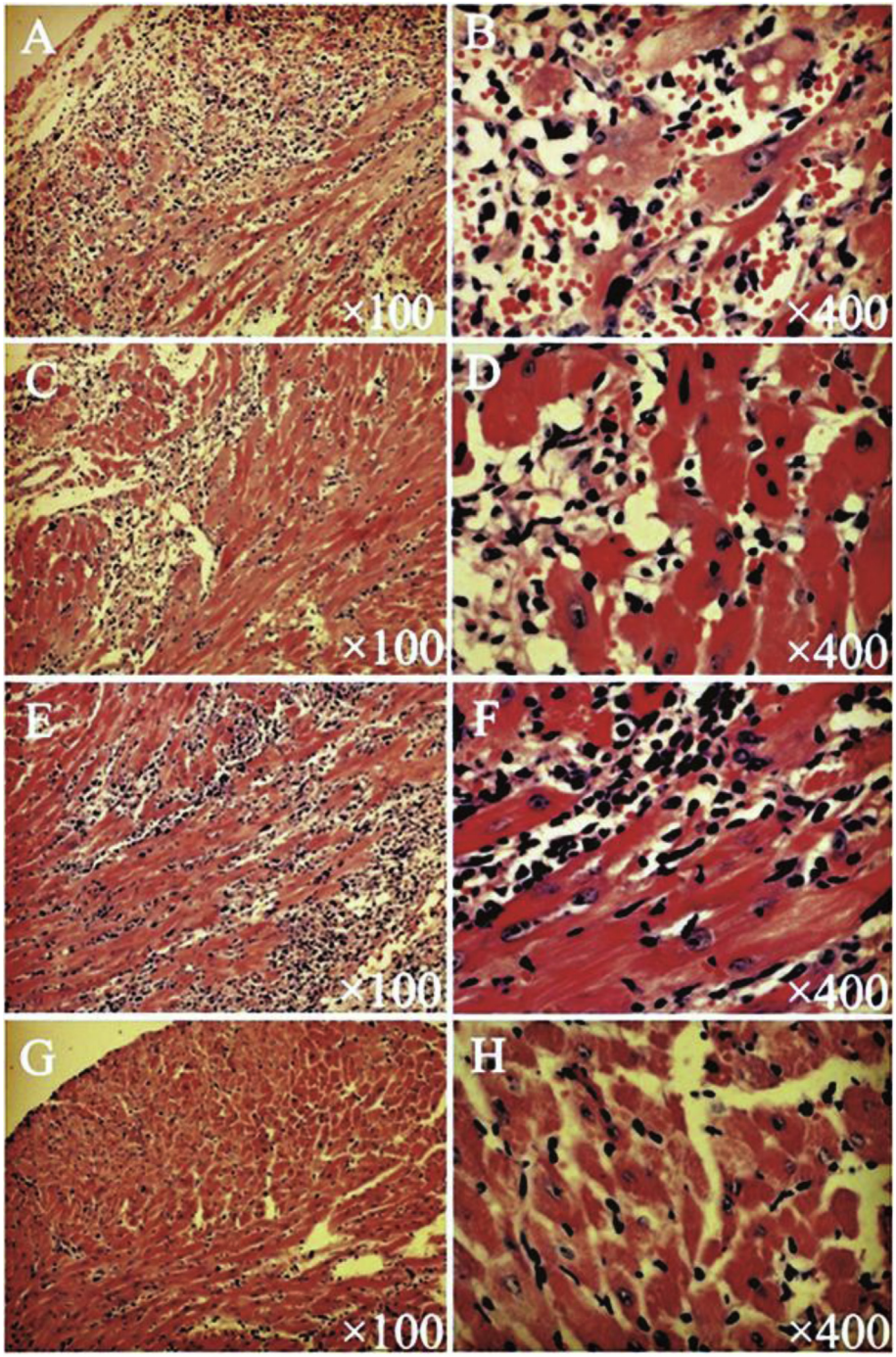

Figure 4. Graft rejection scores in mice received heart transplantation and different preoperative treatments.
Animals were sacrificed on the 7th day after heart transplantation and the bottom of cardiac allografts were collected for H&E staining. Cardiac allograft rejection was evaluated according to the ISHLT scoring system. The control group (R1 group): grade IV rejection, myocardial structure disappeared, and a wide range of vacuoles and lymphocytic infiltration (A, B). The R2 group: IIIB grade rejection, myocardial necrosis, myocardial fiber breakage, multiple fusion of lymphocytic infiltrates (C, D). The R3 group: IIIB grade rejection, myocardial cell necrosis, a large number of inflammatory cell infiltration (E, F). The group R4: IA grade rejection, only a small amount of inflammatory cell infiltration, no myocardial necrosis (G, H).

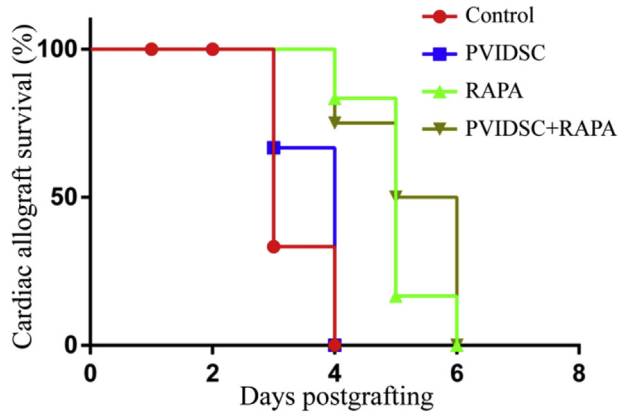

Figure 5. Comparison of the survival time of allogeneic cardiac grafts in mice pre-sensitized with skin grafting (6 animals in each group).
The control group (Rsg1): No preoperative treatments. The Rsg2 group: animals received PVIDSC (0.3 ml, 1×10^7 spleen cells/animal) on the 7th day prior to heart transplantation. The Rsg3 group: animals received intraperitoneal injection of rapamycin (1.5 mg/kg/d) for seven days (from the 7th day prior to heart transplantation to the operation day). The Rsg4 group: animals received a combinative treatment of PVIDSC (0.3 mL, 1×10^7 spleen cells/animal) on the 7th day prior to heart transplantation and intraperitoneal injection of rapamycin (1.5 mg/kg/d) for seven days (from the 7th day prior to heart transplantation to the operation day).

significant differences of the survival time of cardiac allograft were observed among these groups, suggesting that preoperative combination of PVIDSC and rapamycin didn't inhibit cardiac graft rejection mediated by recall responses.

4. Discussion

As early as 30 years ago, it has been observed that donor-specific transfusion (DST) prolonged the survival of organ grafts [7]. DST was then widely used in organ transplantation to prevent graft rejection prior to the advent of the cyclosporine. Recently, the role of DST in extending the survival time of organ grafts has been investigated and confirmed in many animal and clinical experiments. The mechanisms of rejection prevention of DST include clonal deletion [8], chimerism [9], impotence and generation of regulatory T cells [10,11] that induce transformation of Th1 cells to Th2 cells, and regulation of cytokine production [12,13].

Numerous studies [9–11] have demonstrated that portal vein injection of heterologous antigens to recipients is the most efficient way to induce immune tolerance in recipients in organ transplantation. Peripheral vein or intraperitoneal injection of heterologous antigens induce moderate immune tolerance, but subcutaneous injection of heterologous antigens is not as efficient as peripheral vein or intraperitoneal injection of heterologous antigens to induce immune tolerance [7]. It has been reported been portal vein infusion of donor spleen cells and bone marrow cells significantly induced immune tolerance. Donor spleen cells and bone marrow cells reside in the liver, and continue proliferation, lasting their effects of immune tolerance [10]. Spleen cells injected into recipients via peripheral vein were mostly removed after three days of injection, likely by the Kupffer cells of antigen-presenting ability, dendritic cells and liver sinusoids epithelial cells (LSEC) [14]. However, the underlying mechanism is still unclear.

Rapamycin binds to FK506-binding protein (FKBP)-12 to form a complex to inhibit the function of mammalian target of rapamycin (mTOR), affecting the activation of T cells and inducing incompetence of T cells, and regulating the proliferation of T cells [15–20]. *In vitro* experiments have demonstrated that addition of rapamycin to $CD4^+CD25^-$ T cells increased the proliferation of $CD4^+CD25^+FOXP3^+$ regulatory T cells [2]. Combination of rapamycin and IL-2 further increased the proliferation of $CD4^+CD25^+FOXP3^+$ regulatory T cells [3,17]. Our results also shown that preoperative application of rapamycin alone increased the expression of *Foxp3*, but had no effects on the expression of *IL-2*. However, combination of PVIDSC and rapamycin significantly increased the expression of *Foxp3* and *IL-10*. Thus, we concluded that combination of PVIDSC and rapamycin can efficiently induce $CD4^+CD25^+FOXP3^+$ regulatory T cells and IL-10-secreting Tr1.

Previous studies have shown that T-cell receptor (TCR) and IL-2 play a critical role in the proliferation of Treg and sustained expression in peripheral tissues [21]. Calcium blockers such as CNIs, cyclosporine, and, FK-506 are widely used in clinic as immunosuppressants. While these immunosuppressants have effective immunosuppressive function, they reduce the expression of IL-2 and inhibit the proliferation of Treg, thus, do not promote immune tolerance [21–23]. Rapamycin can have both functions.

Our results demonstrated that although combination of PVIDSC and short-term application of rapamycin significantly prolonged the survival of heart grafts in original state mice, rejection of histopathological manifestation was still observed in the R4 group animals. These observations suggest that combination of PVIDSC and rapamycin couldn't completely inhibit cardiac graft rejection. In addition, combination of PVIDSC and short-term application of rapamycin didn't prolong the survival of cardiac grafts in mice pre-sensitized with skin grafting. We speculated that combination of PVIDSC and rapamycin couldn't inhibit the rejection mediated by memory T cells, however, the underlying mechanisms are still unclear.

Generally, the effects of heterologous antigen infusion on the prevention of graft rejection are closely associated with a number of factors including the infusion routs, type and amount of infused cells, and the infusion time [24,25]. Our study indicated the preoperative combination of PVIDSC and short-term application of rapamycin efficiently prolonged the survival of allogeneic cardiac grafts in mice and no post-operative treatments are required. While immune tolerance was not successfully induced by this way, our study provides a novel way for induction of immune tolerance using rapamycin.

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