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Immunotherapeutic strategy based on anti-OX40L and low dose of IL-2 to prolong graft survival in sensitized mice by inducing the generation of $CD4^+$ and $CD8^+$ Tregs

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ABSTRACT

Alloreactive memory cells play a critical role after a second transplant and are difficult to suppress. This study investigated the effect of an immunotherapeutic strategy that combines anti-OX40L, rapamycin (Rapa), and a low dose of IL-2 in a memory cell-based adoptive model. In this model, the median survival time (MST) of the grafts of the combined treatment group was significantly extended compared to that of the control group and other treatment groups. A similar effect was observed regarding a reduction in memory T cells (Tm) and inflammatory cytokines production. Also, the percentages of $Foxp3^+$ regulatory T cells (Tregs) increased in our model. In addition, mounting evidence has shown $CD8^+CD122^+$ T cells are also Tregs. We found that the group of $CD8^+CD122^+PD1^+$ T cells was markedly increased in the combined treatment group, especially in the graft. We further demonstrated that $CD8^+CD122^+PD1^+$ T cells could suppress activated T cells. Our data suggest that anti-OX40L combined with Rapa and a low dose of IL-2 can suppress Tm, modulate CD4 and CD8 Tregs, and induce long-term heart allograft survival in sensitized mice.

1. Introduction

Patients may develop memory cells after exposure to antigens during previous transplantations, blood transfusions, pregnancies, and exposure to pathogens [1,2]. Cells with a memory phenotype are more easily activated than naive cells, and can induce severe allograft rejection through cross-reactivity [3,4]. The OX40-OX40L interaction is critically involved in T cell activation [5]. Both our studies and a large body of other research have reported that anti-OX40L mAbs can inhibit memory cells and prolong graft survival time [6,7]. To increase the beneficial effect in the memory transplant model, anti-OX40L mAbs were used to combine different treatment protocols (e.g. monoclonal antibodies) [8,9]. However, such combinations may be associated with problems of low immunity in recipients due to a suppressed immune response. Based on the important role of Tregs in transplant tolerance, there is a need to identify a combined induction scheme that can induce Tregs and achieve long-term graft survival while also reducing the shock to the recipients' immune system.

Rapa exerts an immunosuppressive effect by blocking the mammalian target of Rapa (mTOR)-mediated signal transduction pathway, and can induce the formation of Tregs and promote their regulatory functions [10,11]. In addition, several studies have shown that IL-2 supports the proliferation and survival of $CD4^+$ Tregs [12–14]. IL-2 is a major T cell growth factor essential for the homeostasis, proliferation, and differentiation of $CD4^+$ and $CD8^+$ T cells. Moreover, IL-2 contributes to the maintenance of the balance between $CD4^+$ Tregs and effector T cells [15,16]. Mice deficient in IL-2 or the IL-2 receptor have reduced numbers of Tregs [17]. In both clinical studies and animal models (e.g., in the treatment of autoimmune and inflammatory diseases), a low dose of IL-2 has been shown to selectively activate $CD4^+$ Treg subsets, and the production of cytokines involved in suppressive functions (e.g., IL-10 and TGF-β) are also increased in response to IL-2 therapy [18–20]. In organ transplant and GVHD models, a low dose of IL-2 combined with Rapa can significantly delay transplant rejection and reduce GVHD, in association with an increased number of Tregs in mice [21,22].

IL-2 can also affect $CD8^+$ T cells and $CD8^+Foxp3^+$ Tregs [23,24]. IL-2

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with increased CD122 affinity can preferentially expand CTLs, as well as increase $CD8^+CD122^+$ Tregs. $CD8^+CD122^+$ Tregs can correspond to $CD4^+CD25^+$ Tregs, given that CD122 is the β subunit of the IL-2R, whereas CD25 is the α subunit of IL-2R [25]. Recent findings indicate that $CD8^+CD122^+$ Tregs can suppress autoimmunity, rejection, and vaccine-induced antitumor immune responses similar to $CD4^+CD25^+$ Tregs, in mouse models [26–28]. PD-1 is a negative costimulatory molecule critical for the suppression of autoimmunity and the induction of tolerance. It has previously been reported that PD-1 expression on $CD8^+$ T cells is responsible for their exhaustion and a PD-1 blockade was found to restore their function [29]. Thus, some studies use PD-1 as an important biomarker to distinguish between memory and regulatory $CD8^+CD122^+$ T cells [30]. Moreover, $CD8^+CD122^+PD1^+$ T cells may play a critical role in the balance between long-term immunity and tolerance as classical $CD4^+CD25^+Foxp3^+$ Tregs.

2. Materials and methods

2.1. Mice

Male C57BL/6 (H-2^b), BALB/c (H-2^d), C3H (H-2^k), and $Rag1^{-/-}$ mice (B6 background) (8–12-weeks old) were purchased from Jinan Pengyue Experimental Animal Breeding Co. Ltd. (Shandong, China) and Beijing Vital River Laboratory Animal Technologies Co. Ltd. (Beijing, China). This study was carried out in strict accordance with the guidelines established by the Institutional Animal Care and Use Committee of the Chinese Association for Laboratory Animal Sciences.

2.2. Establishing memory, $Foxp3^+$ Treg depletion and $CD8^+CD122^+PD1^+$ T cell transfer in mouse heart transplant models

Vascularized heterotopic heart transplant from BALB/c to B6 mice was performed by anastomosis to the vessels of the neck using a non-suture cuff technique. Both the donor and recipient mice were anesthetized by an intraperitoneal injection with sodium pentobarbital (1.5 mg/kg). Graft survival was monitored twice daily. Rejection was defined as the loss of palpable cardiac contractions.

Full-thickness skin grafts from BALB/c mice were prepared from the lateral thoracic skin (approximately 1 cm^2 in area), and grafted onto the lumbar region of the B6 mice. After four weeks, the percentage of CD4 and CD8 Tm, total alloantibodies, and the alloantibody titer were assessed, and the splenocytes were isolated from the B6 skin graft recipients. The cells were counted and viability was determined by trypan blue exclusion. Viability was required to exceed 90% for the splenocytes to be transferred. The isolated splenocytes (5×10^6 cells) were adoptively transferred to B6 recipients via the vein the day before heart transplantation.

$CD4^+CD25^+Foxp3^+$ Tregs were depleted using an anti-CD25 antibody (PC61, BioExpress, USA). The PC61 depleting antibody was intraperitoneally injected (300 μg/mouse) into the long-term survival mice to deplete Treg cells.

$CD8^+CD122^+PD1^+$ or $CD8^+CD122^+PD1^-$ cells (5×10^3 cells) were isolated from the long-term survival donor hearts combined with naïve T effector cells (1×10^6 cells from the spleen) were injected into $Rag1^{-/-}$ mice on day-1 before heart transplantation.

2.3. Flow cytometry

Anti-CD4 Alexa700 (RM4-5), anti-CD8a APC-cy7 (53–6.7), anti-CD25 PE-cy7 (PC61), anti-CD44 eFlour450 (pacific blue) (IM7), anti-CD62L Percp-cy5.5 (MEL-14), anti-CD62L PE (MEL-14), anti-CD3 APC (17A2), anti-CD122 FITC (5H4), anti-PD-1 eFlour450 (RMP1-30), FC block (CD16/CD32) (2.4G2), and isotype controls were purchased from BD Pharmingen (San Jose, CA, USA) and eBioscience (San Diego, CA, USA).

Tregs were labeled using a Mouse Regulatory T cell staining kit purchased from eBioscience (Cat No. 88-8111). A cytometric bead array (CBA) was performed using commercially available kits (BD Biosciences) to detect the levels of IFN-γ, IL-2, IL-4, IL-6, IL-17, and IL-10. Stained cells and the CBA were analyzed on a flow cytometer (BD FACSCalibur) and the data were analyzed using FlowJo 7.5 software.

2.4. Immunotherapy treatment of cardiac graft recipients

B6 mice that received a heart transplant were treated with one of the following four immunotherapeutic approaches: 1) low dose recombinant human IL-2 (Proleukin, Novartis, 5000 IU daily from day 0 to day 14); 2) Rapa (1.5 mg/kg daily from day 0 to day 10); 3) Rapa + anti-OX40L (a-OX40L, RM134L, 0.25 mg on days 0, 2, 4, and 6); and 4) Rapa + IL-2 + a-OX40L. IL-2, Rapa, and a-OX40L were administered into the abdominal cavity. The negative control group was administered PBS. Samples from recipient mice was collected on day 5 (naïve control, Tm control and IL-2 groups), day 15 (Rapa, Rapa + a-OX40L and Rapa + IL-2 + a-OX40L groups), and day100 (Rapa + IL-2 + a-OX40L long term survival mice) post-transplant.

2.5. Histological evaluation of rejection

The heart grafts were removed at various time points post-transplantation. Paraffin-embedded transventricular tissue sections (5-μm thick) were stained with hematoxylin and eosin (H&E). Rejection was graded based on the extent of lymphocytic infiltration and the anatomical localization of leukocytes according to the International Society of Heart and Lung Transplantation (ISHLT) standards in a blinded fashion by an experienced pathologist.

2.6. Lymphocyte extraction from cardiac allografts

Cardiac allografts were removed, minced with a sterile blade, and incubated in 10 mL HBSS with 2 mg/mL collagenase II at 37 °C for 2 h. The incubation was stopped by the addition of PBS (containing 10% fetal calf serum), and the cells were strained through a 70-μm nylon strainer. Subsequently, cells were centrifuged and resuspended in 2 mL DMEM. The lymphocytes were isolated from these cells using lymphocyte separation medium (3 mL per cardiac allograft) via density gradient centrifugation. The isolated lymphocytes were resuspended in 300 μL FACS buffer for flow cytometric analysis.

2.7. Mixed lymphocyte reactions

The spleens were processed into single-cell suspensions, labeled with CFSE (Sigma-Aldrich). Next, 50×10^6 CFSE-labeled splenocytes were plated on a petri dish for 45 min at 37 °C to enrich for lymphocytes. After 45 min, the nonadherent cells were collected, washed, and incubated (1×10^5 cells) with irradiated (2000 cGy, 1×10^5 cells) naive BALB/c splenocytes in 96-well U-bottom titration plates in MLR medium. Irradiated syngeneic cells and C3H third-party splenocytes were used as controls. After four days, the cells were stained with fluorochrome-labeled Abs against mouse CD4, CD8, and 7AAD (BD Biosciences) followed by an analysis by flow cytometry. The data were analyzed using FlowJo software.

2.8. Statistical analyses

Survival curves and graphs of the histological data were created using GraphPad Prism® (GraphPad software, La Jolla, CA, USA). The survival curves were analyzed using a log-rank test. Data from the flow cytometry and CBA experiments were analyzed using a one-way analysis of variance and expressed as the means ± standard deviation (SD). A value of $P < 0.05$ was considered to be statistically significant and $P < 0.01$ was highly statistically significant.

3. Results

3.1. Anti-OX40L combined with a low dose of IL-2 and Rapa prolongs the survival time of cardiac grafts in a memory transplant model

B6 mice that received BALB/c cardiac grafts and adoptively transferred spleen cells from alloantigen-primed B6 mice (memory model) exhibited accelerated graft rejection (naïve control group, no cell transfer, MST = 7.7 d vs. Tm control group, allo-primed cell transfer, MST = 5.2 d). In the memory model, treatment with Rapa alone could prolong the MST to 15.3 days, whereas treatment IL-2 alone could not effectively extend the MST. Combined treatment with Rapa and a-OX40L could markedly extend the graft survival time. When a low dose of IL-2 was added to the Rapa and a-OX40L treatment, cardiac graft rejection was substantially delayed. In addition, approximately 60% of the cardiac grafts survived for longer than 100 days in the recipients that received triple therapy (Fig. 1A). Next, the long-term survival mice (day 100 after heart transplantation) were used as recipients for transplanted BALB/c (donor) or C3H (third party) skin. Approximately 80% percent of the BALB/c skin could survive for longer than 60 days. In comparison, the C3H skin grafts were rejected rapidly following transplantation (6–8 days) (Fig. 1B).

3.2. Inflammatory cell infiltration in the grafts

Next, we compared the level of gross immunopathology in the control and treatment groups (n = 6 mice/group). Representative examples of cardiac graft tissue are presented in Fig. 2A. The degree of damage was reported as an ISHLT score (Fig. 2B). The control group displayed varying degrees of cellular infiltration and damage, with ISHLT scores ranging from level 3 to 4. Treatment with Rapa and a-OX40L, both alone or in combination, was found to reduce the ISHLT scores. The ISHLT score in mice that received triple therapy was significantly lower than that in the mice that received other treatment protocols (Fig. 2B).

3.3. Alloreactivity of T cells in the recipients

To assay the alloreactivity of T cells in the recipients, the spleens were harvested when grafts rejected and at day 100 (LT group) after transplantation. The T cells were isolated and prepared for analysis using an MLR assay (Fig. 3A and B). As shown in Fig. 3C, compared with the Tm control group, T cell proliferation was inhibited by treatment with Rapa alone. Combination treatment with Rapa and anti-OX40L resulted in a much lower $CD4^+$ T cell response. When IL-2 was added, the response of CD8 cells were further inhibited compared to that of the Rapa group (Fig. 3D). Both the alloreactivity of $CD4^+$ and $CD8^+$ T cells remained low at levels on day 100, but retained normal reactivity to a third party mouse (Fig. 3C and D).

3.4. Percentage of $Foxp3^+$ Tregs in the spleen, DLN, and heart

Spleens, DLNs and grafts were harvested on day 15 and day 100 (LT group) after transplantation. On day 15, the percentage of $Foxp3^+$ lymphocytes in the spleen, DLN, and graft increased in the Rapa group (Fig. 4A - C), whereas the percentage was only increased in the DLN of the IL-2 treatment group, (Fig. 4B). In the Rapa + a-OX40L + IL-2 group, the percentage of $Foxp3^+$ lymphocytes was further increased in the DLN and heart. Even on day 100, the percentage of $Foxp3^+$ Tregs remained at high levels in the Rapa + a-OX40L + IL-2 group. The critical role of Tregs was confirmed by the observation that the grafts in the long-term

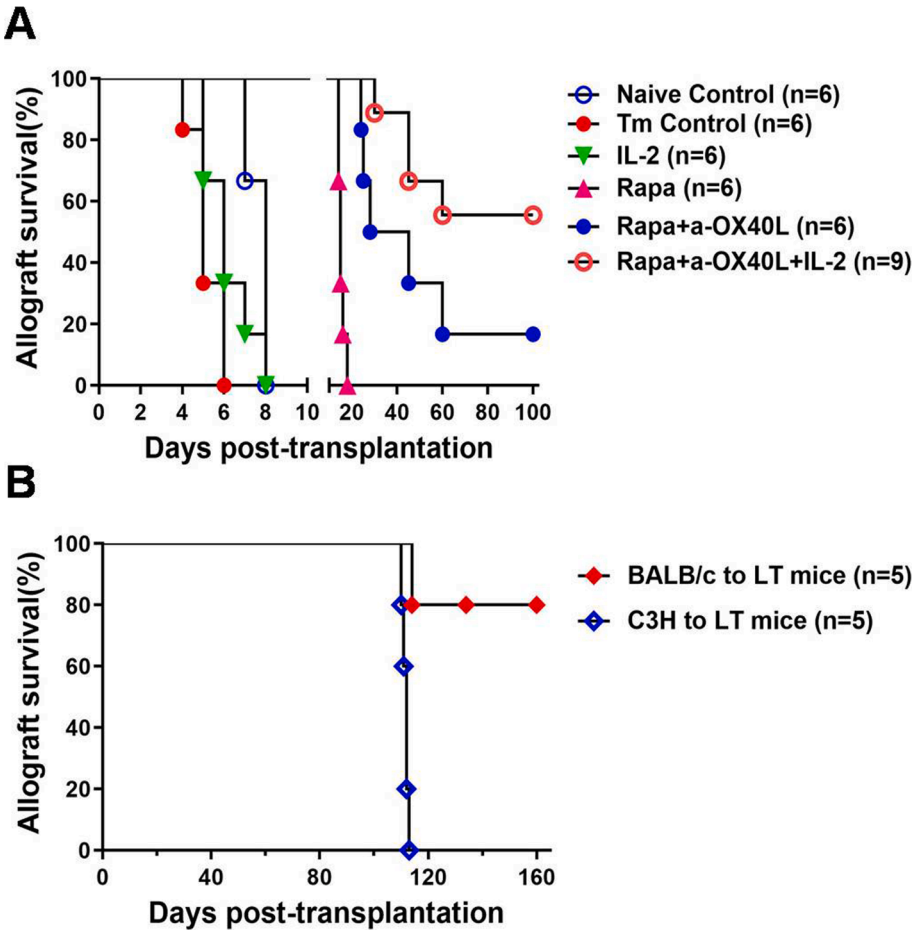

Fig. 1. Anti-OX40L combined with Rapa and a low dose of IL-2 significantly prolongs the survival time of cardiac grafts in sensitized mice. (A) Sensitized B6 mice were transplanted with BALB/c cardiac allografts and divided into different treatment groups. In the group treated with anti-OX40L combined with Rapa and low dose IL-2, approximately 60% of the cardiac grafts survived for longer than 100 days in the recipients (n = 6–9 mice per group). (B) BALB/c (donor) or C3H (third party) skin was transplanted to long-term surviving mice. About 80% percent of the BALB/c skin could survive longer than 60 days, whereas the C3H skin grafts were rejected within 6–8 days post-transplantation. MSTs were analyzed using the Kaplan-Meier method (n = 5 mice per group).

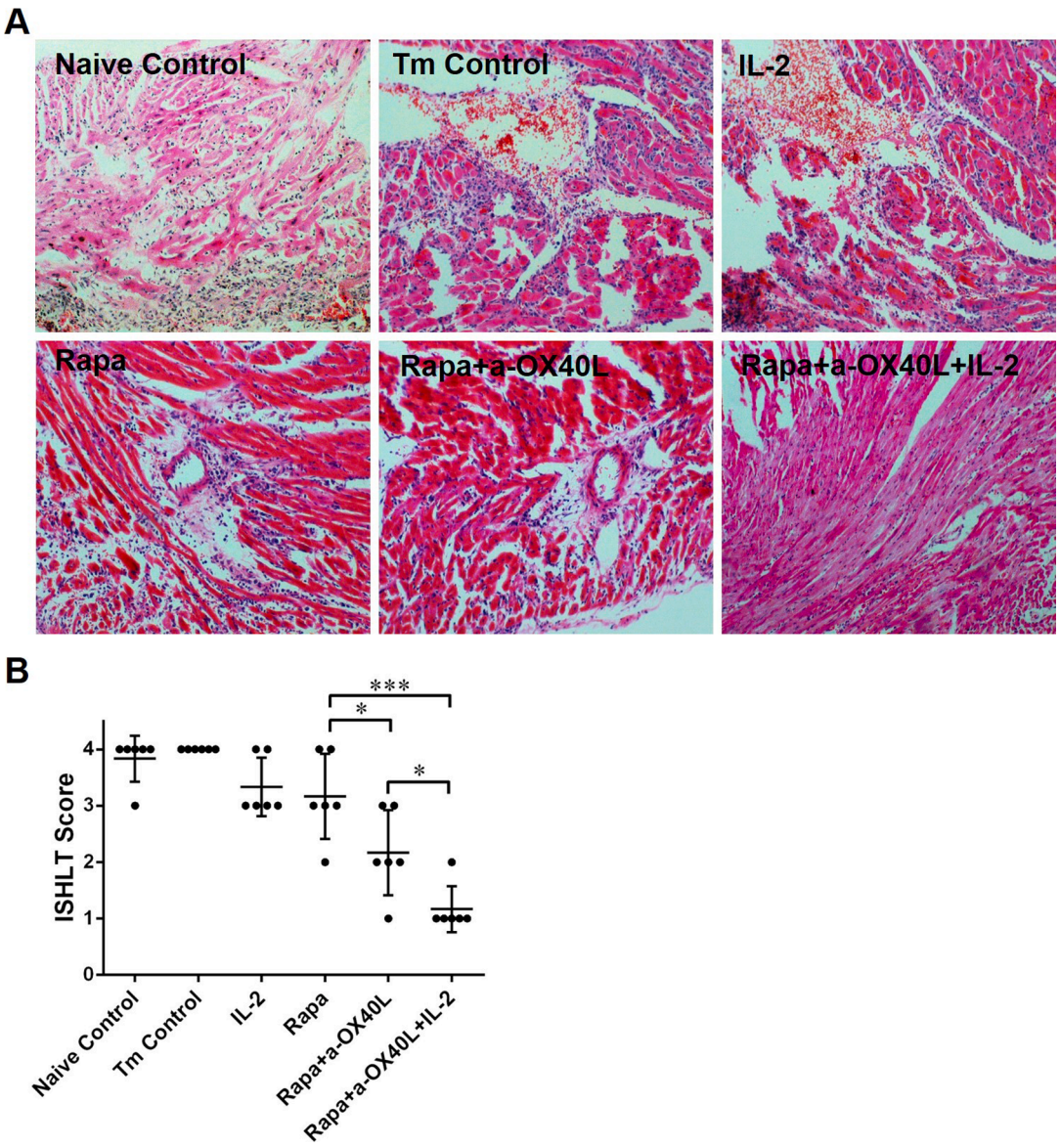

Fig. 2. Pathological changes and the ISHLT scores. (A) Grafts were detected with H&E staining for different treatment groups (100 ×). (B) ISHLT scores and statistical analyses of the sections. Each dot on the graph represents the score for each animal in the indicated group (n = 6 mice per group). The line represents the mean scores (*P < 0.05; ***P < 0.001).

survival group were rejected in a short period of time (within two weeks) when the Foxp3$^+$ Tregs were depleted (Fig. 4D).

3.5. Cytokines in the recipient serum

The levels of immunomodulatory cytokines in the serum of the recipient mice were assessed. The group treatment with Rapa was found to reduce IFN-γ and IL-2 (Fig. 5A and B), whereas no effect was observed in the group treated with IL-2 alone. In the Rapa + a-OX40L + IL-2 group, the secretion of IFN-γ, IL-2, IL-6, and IL-17 were further inhibited compared to the group treated with Rapa alone (Fig. 5A, B, D, and E). In the long-term group, these cytokines remained at low levels. IL-10 has been established as an important inhibitory cytokine. Rapa + a-OX40L treatment could increase the secretion of IL-10, which was further enhanced when IL-2 was added (Fig. 5F).

3.6. Relationship between rejection and the level of CD8$^+$CD122$^+$PD1$^+$ T cells in spleen, DLN, and heart

We examined the CD8$^+$CD122$^+$PD1$^+$ T cells in spleen, DLN, and heart. The percentage and absolute number of CD8$^+$CD122$^+$PD1$^+$ T cells in the graft increased in the IL-2 only and Rapa + a-OX40L + IL-2 groups (Fig. 6B - D). In the long-term survival group, CD8$^+$CD122$^+$PD1$^+$ T cells were maintained at high levels in the DLN and graft. To identify the cell populations that were potentially involved in the suppression of graft rejection, CD8$^+$CD122$^+$PD1$^+$ and CD8$^+$CD122$^+$PD1$^-$ T cells were sorted from the long-term survival grafts and adoptively transferred with Teff cells into the transplant model. CD8$^+$CD122$^+$PD1$^+$ T cells were able to suppress graft rejection and extend the MST of grafts from 18.6 days to 38 days; however, the same effect was not observed for CD8$^+$CD122$^+$PD1$^-$ T cells (Fig. 6E).

4. Discussion

The OX40-OX40L interaction has previously been demonstrated to

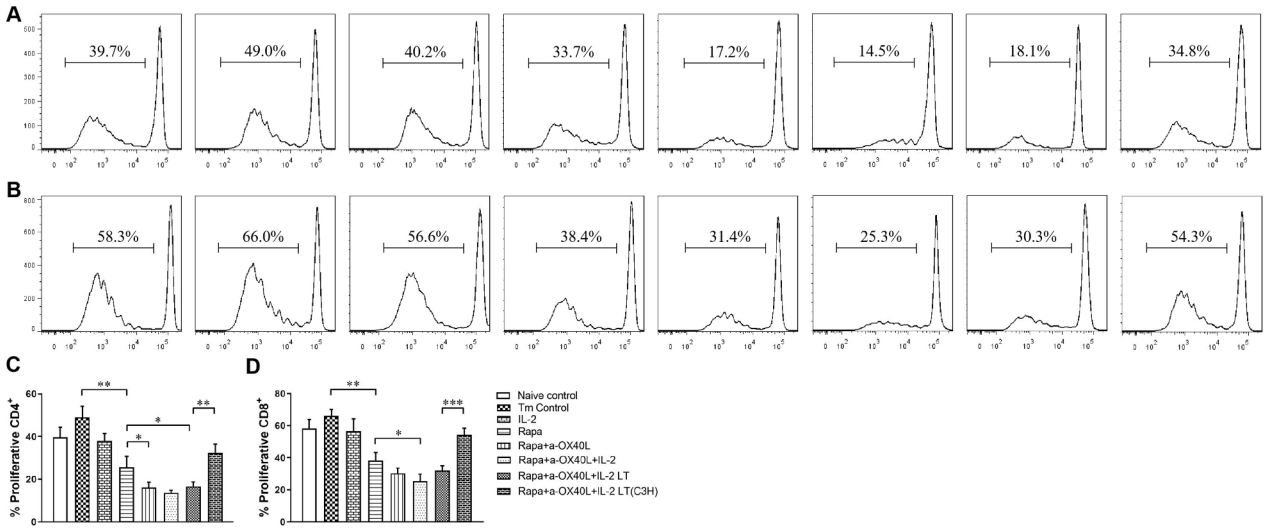

Fig. 3. Proliferation of CD4$^+$ and CD8$^+$ T cells in recipient mice. T cell proliferation was prepared for analysis using an MLR assay. (A and B) Percentage of the proliferating CD4$^+$ (A) and CD8$^+$ (B) T cells in different treatment groups. (C and D) Statistical analyses of proliferative CD4$^+$ (C) and CD8$^+$ (D) T cells in different BALB/c stimulate groups and C3H third party stimulate group. (*P < 0.05; **P < 0.01; ***P < 0.001).

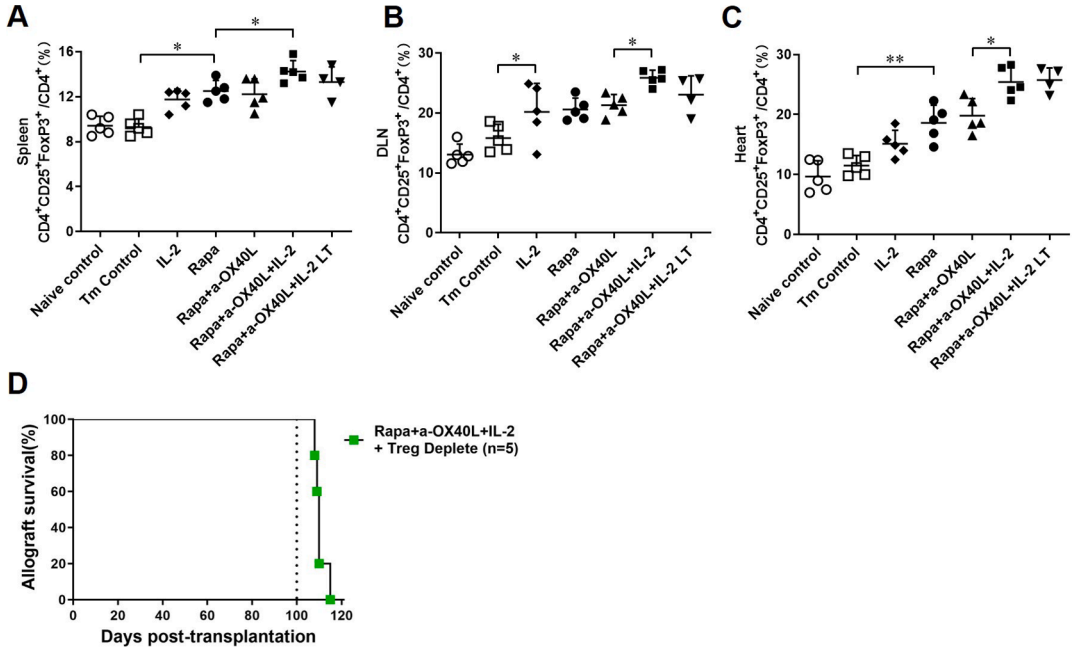

Fig. 4. Graft rejection occurs rapidly in recipients following Treg depletion. (A - C) Statistical analysis of the proportion of Tregs in the spleen (A), DLNs (B), and hearts (C) (n = 4–5 mice per group). (D) Tregs were depleted in the long-term survival mice. All of the grafts were rejected within two weeks (n = 5). (*P < 0.05; **P < 0.01).

play a pivotal role in the generation and activation of memory T cells. Moreover, blocking OX40L using mAbs can effectively inhibit Tm cells. In our previous study, we used the combination of donor-specific transfusion, Rapa, and a-OX40L to prolong the MST of cardiac grafts in an alloantigen primed mice heart transplant model, suppress CD4$^+$ and CD8$^+$ Tm cell activation, and increase the number of Tregs [6]. In addition, IL-2 is important for the survival and expansion of Tregs. Previous studies have found that a low dose of IL-2 selectively activates CD4$^+$Foxp3$^+$ Treg subsets [31]. For example, a low dose of IL-2 combined with Rapa could promote allogeneic skin graft survival in mice [21], and the delivery of a low dose of IL-2 alone or combined with Rapa was found to prevent hyperglycemia in NOD mice [32,33]. In nonhuman primates, a low dose IL-2 expanded CD4$^+$ and CD8$^+$ regulatory T cells in vivo [34]. Additionally, a low dose IL-2 restored Treg homeostasis in patients with chronic graft-versus-host disease [22]. In these studies, the dose of IL-2 was found to be extremely important in the Treg-inducing process. The definition of low dose IL-2 was not the same in different diseases and models. For example, 0.33–3 million IU/d IL-2 was used for human GVHD, T1D and SLE treatment, and in the mice model about 10^2 to 10^4 IU/d/per mouse was used for different disease models [35–38]. In contrast to a low dose, a high dose of IL-2 limited the expansion of Tregs, but also participated in the development and progression of some diseases [18,39].

Based on this research, a-OX40L, Rapa, and a low dose of IL-2 were used to suppress rejection in a memory transplant model. The MST of the cardiac grafts was substantially extended and approximately 60% of the cardiac grafts survived for longer than 100 days (Fig. 1A). Sequential skin transplantation to the long-term survival mice revealed that the

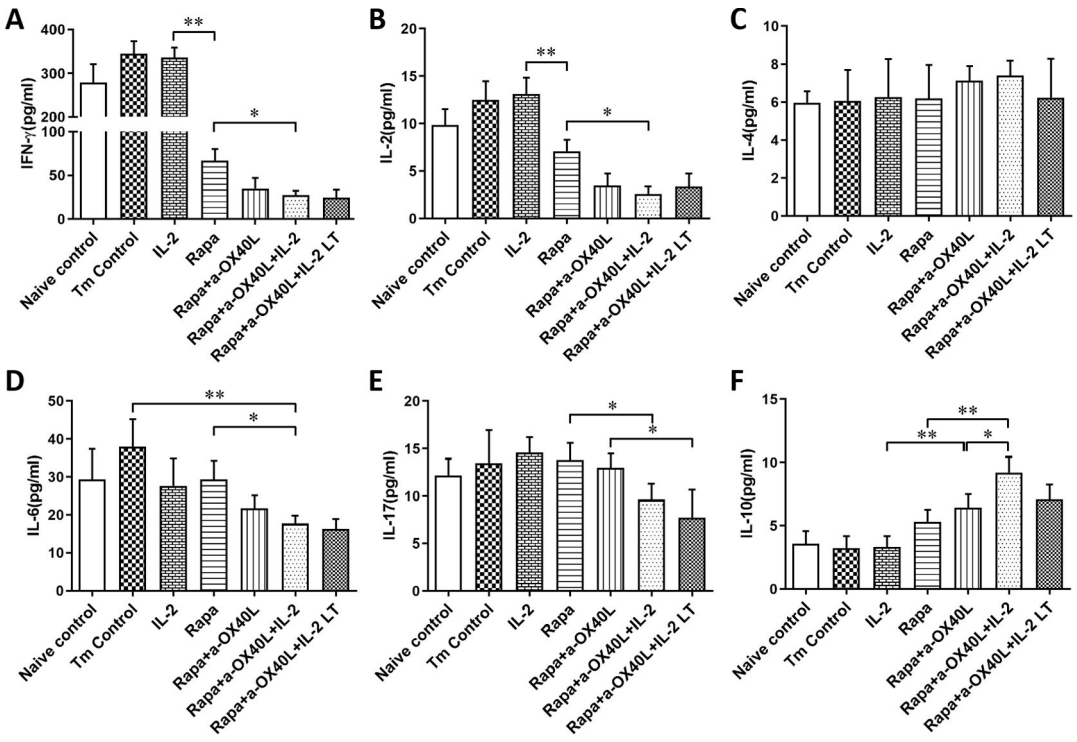

Fig. 5. Serum cytokine levels in the recipients. (A - F) The level of IFN-γ, IL-2, IL-4, IL-6, IL-17, and IL-10 in the recipients' sera. Data are representative of three independent experiments and presented as the mean proportion (*P < 0.05; **P < 0.01).

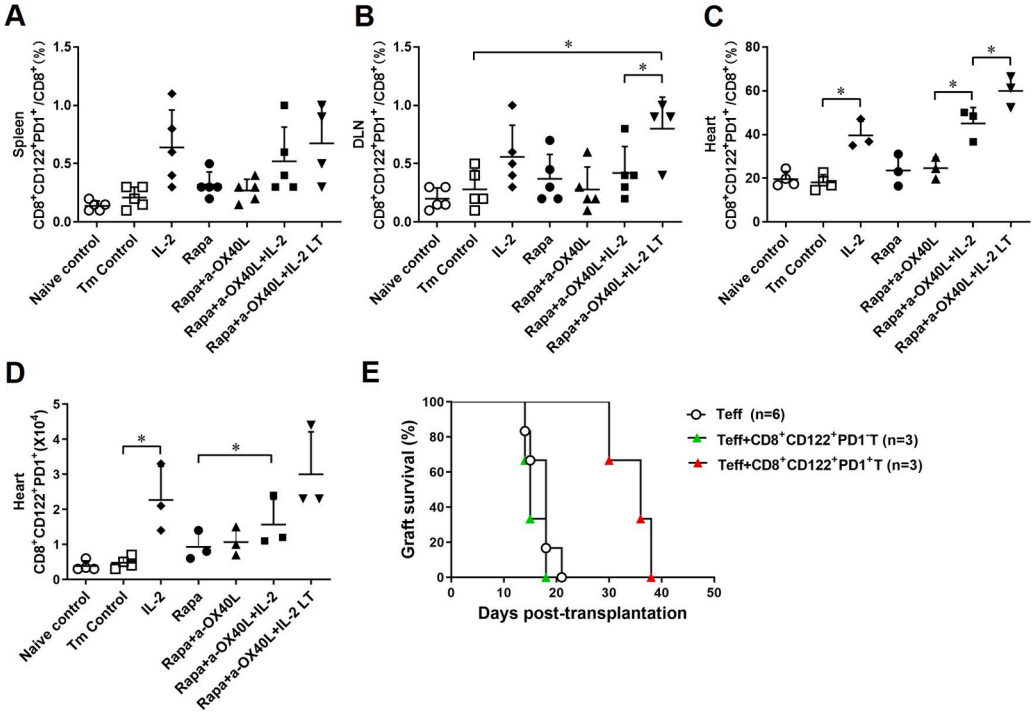

Fig. 6. Level of $CD8^+CD122^+PD1^+$ T cells in the spleens, DLNs and hearts, and associated effect on suppressing graft rejection. (A - C) The percentage of $CD8^+CD122^+PD1^+$ T cells in the spleens (A), DLNs (B), and hearts (C). (D) The absolute number of $CD8^+CD122^+PD1^+$ T cells in the grafts (*P < 0.05). (E) $CD8^+CD122^+PD1^+$ T and $CD8^+CD122^+PD1^-$ T cells were sorted from the long-term survival grafts and adoptively transferred with Teff cells into the transplant model. The MST of the $CD8^+CD122^+PD1^+$ T cell group was extended to 38 days (n = 3); however, the MST of the $CD8^+CD122^+PD1^-$ T cell group was similar to that of the control group.

recipients were tolerant to the donors but still had a normal immune response to third party antigen (Fig. 1B). This donor-specific immune tolerance was also verified by MLR, as both $CD4^+$ and $CD8^+$ T cells exhibited low proliferation in the long-term survival group when stimulated by BALB/c cells compared to the C3H group (Fig. 3). Mice treated with the triple therapy exhibited decreased inflammatory cell infiltration, and achieved the lowest ISHLT score (Fig. 2). $CD4^+$ or $CD8^+$ Tm cells displayed the same trend in the PBMCs, spleens, and DLN, and the percentages were maintained at low levels in the long-term survival group compared to the other treatment groups (Supplemental Fig. 1). Moreover, the level of Th1 cytokines, IL-2 and IFN-γ, were reduced in all the treatment groups except the IL-2 treatment group. In addition to Th1 cytokines, IL-6 and IL-17 were also decreased in the combined treatment groups. This finding indicated that the protocol affected T cell

differentiation. IL-10 was substantially increased in the anti-OX40L, Rapa, and low dose IL-2 group (Fig. 5). IL-10 is a pleiotropic cytokine that can also initiate a switch between Th1 and Th2 responses, as well as function as a well-established inhibitory factor [40,41].

Tregs can also be induced by IL-10 [42,43]. Several publications have shown that a low dose of IL-2 may have selective effects on Treg cells without stimulating effector cells in both research and clinical studies [20,44,45]. In our protocol, when treatment was combined with a low dose of IL-2, the percentage of $Foxp3^+$ Tregs further increased in the DLN and heart compared to the group treated with anti-OX40L and Rapa. A high percentage of $Foxp3^+$ Tregs was maintained a in the Rapa + a-OX40L + IL-2 group in the long-time survival recipients (Fig. 4A - C). This data further confirms that a low dose of IL-2 selectively activated a subset of $Foxp3^+$ Tregs. When $Foxp3^+$ Tregs were depleted, the grafts in the long-term survival group were rapidly rejected (Fig. 4D). The increased percentage of Tregs in the DLNs and grafts suggests that local immunomodulatory effects were critical for graft longevity. Low dose of IL-2 not only could activate the subset of $Foxp3^+$ Tregs, and Takeru Asano etal. found that IL-2-induced Treg proliferation was promptly followed by increased PD-1 expression [46]. In this study, we also found that the percentage and absolute number of $CD8^+CD122^+PD1^+$ T cells were increased in the Rapa + a-OX40L + IL-2 group, especially in the heart (Fig. 6C). Moreover, the percentages were further increased in the DLNs and grafts of the long-term survival recipients. Suzuki's group were the first to report that $CD8^+CD122^+$ T cells maintain T cell homeostasis, and many studies have demonstrated that $CD8^+CD122^+$ T cells are Tregs which can suppress the T cell response and regulate various autoimmune diseases [26,47,48]. The study by Dai et al. further demonstrated that $CD8^+CD122^+$ T cells contained a substantial subset of $PD\text{-}1^+$ cells that were Tregs [49], whereas $CD8^+CD122^+PD1^-$ T cells were memory T cells. Moreover, a previous study demonstrated that this group cells could promote the development of diabetes in the NOD mice [29]. In addition, $CD8^+CD122^+PD1^+$ T cells could produce a greater quantity of IL-10 than the $PD1^-$ T cells, and the suppression of this group of Tregs was partially dependent on the production of IL-10 [50]. In our study, we established an adoptive transfer model to elucidate whether $CD8^+CD122^+PD1^+$ T cells can suppress graft rejection compared to the $CD8^+CD122^+PD1^-$ T cell group and Teff group (Fig. 6D). Together, these data provide further support that $CD8^+CD122^+PD1^+$ T cells represent a novel group of Tregs.

In summary, the Rapa, a-OX40L, and low dose of IL-2 protocol significantly prolonged the MST of cardiac grafts in a memory transplant model. A low dose of IL-2 induced Tregs production and regulated the immune response in our memory transplant model. Moreover, $CD8^+CD122^+PD1^+$ T cells, as a novel group Tregs were also found to play a critical role, and may provide a new target for inducing tolerance.

Animal ethics

This study was carried out in strict accordance with the guidelines established by the Institutional Animal Care and Use Committee of the Chinese Association for Laboratory Animal Sciences.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2021.107663.

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