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Ethylene carbodiimide-fixed donor splenocytes combined with cordycepin induce long-term protection to mice cardiac allografts

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ABSTRACT

Infusion of ethylene carbodiimide-fixed donor splenocytes (ECDI-SPs) is an effective method to induce donor-specific protection to allografts. However, the ischemia reperfusion (I/R) injury during transplant leads to abundant of pro-inflammatory cytokines, which negates the effect of ECDI-SPs. Therefore, suppressing pro-inflammatory cytokine secretion while promoting anti-inflammatory cytokine release would enhance the graft protective efficacy of ECDI-SPs. In this study, we aimed to determine the effect of ECDI-SPs combined with a short course of cordycepin (an anti-inflammatory agent) on the long-term outcomes of mice cardiac allografts. Our results demonstrated that ECDI-SPs combined with cordycepin significantly promoted mice cardiac allograft survival compared with ECDI-SPs monotherapy. This effect was accompanied by decreased production of pro-inflammatory cytokines (IL-1β, IL-6, IL-17 and TNFα), increased secretion of anti-inflammatory cytokines (IL-10 and TGFβ), inhibition of Th17 and expansion of Tregs, and prevention of I/R injury. We concluded that cordycepin appeared to enhance the effect of modulating cytokine profile and regulate the Teff:Treg balance so as to strengthen the graft protective effect of ECDI-SPs. Our study of ECDI-SPs combined with cordycepin may provide a promising approach for prolong allograft survival.

1. Introduction

Organ transplantation is considered as an effective therapy to treat end-stage organ failure. However, despite medical and surgical advances, acute and chronic rejection remains the bottleneck to achieve long-term graft and patient survival. Although rejection episodes could be readily treated by the use of immunosuppressants, these agents are usually associating with lifelong side-effects, such as infections, organ toxicities, metabolic disorders and malignancies [1,2]. Therefore, inducing immune tolerance is still considered as the best method to both prevent allograft rejection and obviate the need for continuous use of immunosuppressants.

Recently, studies reveal that apoptotic cells display potent anti-inflammatory and immunoregulatory effects [3]. Administration of apoptotic cells carrying donor MHC molecules have been reported to help preventing transplant rejection in many animal models [3,4]. As a water-soluble chemical, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (ECDI) has been used as a cross-linker for conjugating peptides to cellular membranes [5]. Intravenous infusion of ECDI-fixed donor splenocytes (ECDI-SPs) effectively and safely induce antigen-specific tolerance *in vivo* [6,7]. ECDI-SPs have also been used to suppress autoimmune diseases such as mice experimental autoimmune encephalomyelitis and mice autoimmune diabetes [8,9]. Recent studies show that ECDI-SPs prolong mouse cardiac and vascularized skin allograft survival [10,11] and induce donor-specific immune tolerance in mouse islet cell transplant [12]. The mechanism of ECDI-SPs treatment involves deletion and anergy of T cell, inhibition of effector T cells (Teff) and expansion of regulatory T cells (Tregs) [6,10,13]. However, the establishment and maintenance of a favorable Teff:Treg balance is affected by the microenvironment of the allograft [12]. Pro-inflammatory cytokines weaken the protective role of ECDI-SPs by promoting naïve $CD4^+$ T cells to differentiate into pathogenic Teff and by preventing Tregs reconstitution [6,14]. This may be the reason that ECDI-SPs monotherapy induce only short-term graft protection for solid

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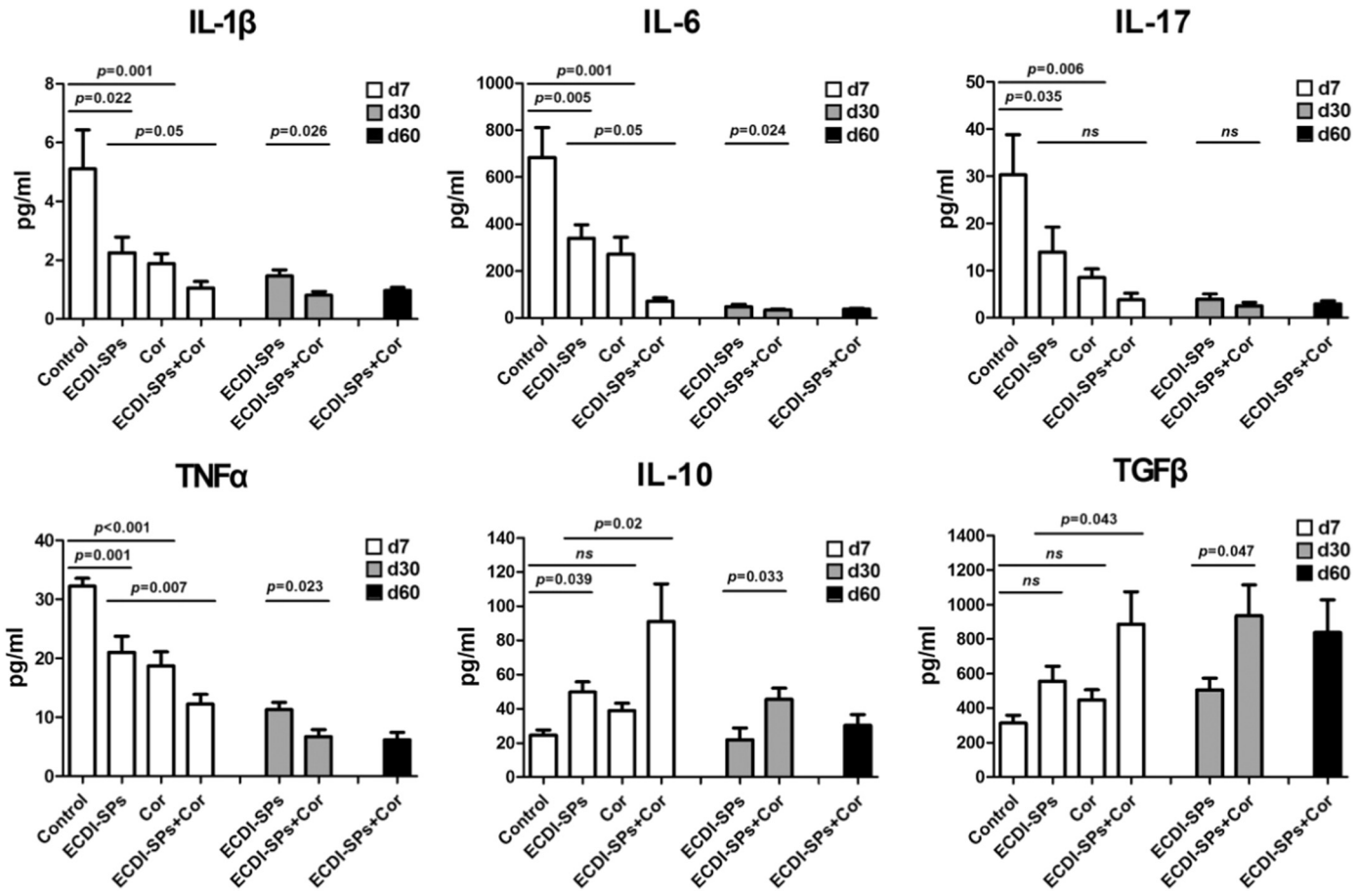

Fig. 1. ECDI-SPs combined with cordycepin exerted an additive effect on reducing pro-inflammatory cytokines and increasing anti-inflammatory cytokines. Serum cytokine levels were measured by ELISA kits at day 7, day 30, and day 60 after transplantation. Data were represented as median ± SD from 5 to 6 recipients in each group. One-way ANOVA was used for analysis between multiple groups and Student's *t*-test was used for analysis between two groups. ECDI-SPs, ECDI-treated donor splenocytes; Cor, cordycepin.

organ transplantation. Thus, inhibiting pro-inflammatory cytokine production while at the same time promoting anti-inflammatory cytokine release may create an appropriate support milieu to enhance the graft-protective efficacy of ECDI-SPs.

Cordycepin, also named 3′-deoxyadenosine, is an active component isolated from Cordyceps militaris. Cordycepin has potent anti-thrombotic, anti-oxidant and anti-inflammatory properties. In lipopolysaccharides-induced microglia cells, cordycepin attenuates the release of pro-inflammatory mediators, such as NO, PGE2, IL-1β and TNF-α [15]. In lipopolysaccharides-stimulated macrophages, cordycepin inhibits M1 cytokines (IL-1β and TNF-α) gene expression whereas increased M2 cytokines (IL-10, IL-1Ra, and TGF-β) gene expression [16]. In human peripheral blood mononuclear cells, cordycepin promotes the expression of IL-10 protein [17]. Recent studies reveal that cordycepin exerts potent protective functions against cerebral and heart ischemia reperfusion (I/R) injury [18–20]. In addition, cordycepin has been use as an adjunctive treatment by immunosuppressive therapy in renal transplantation [21].

Therefore, we speculated that cordycepin-infused peri-transplant would shift the cytokine profile from pro-inflammatory to anti-inflammatory and help to create a support milieu to strengthen the protective function for ECDI-SPs. In this study, using a mouse cardiac transplant model, we aim to verify the hypothesis that infusion of ECDI-SPs combined with a peri-transplant short-term course of cordycepin would promote long-term graft survival.

2. Materials and methods

2.1. Animals

Six to eight weeks old Male BALB/c, C57BL/6, and C3H mice weighing 20 to 25 g were purchased from the Experimental Animal Center of Sun Yat-sen University. All animals were housed under specific pathogen-free (SPF) conditions and were supplied by Experimental Animal Center of Guangzhou Medical University. All animal experiments conform to the institutional guidelines of Guangdong Province and were approved by the Use Committee for Animal Care and the Guangzhou Medical University Institute Research Ethics Committee.

2.2. Abdominal heterotopic cardiac transplantation

Abdominal heterotopic cardiac transplantation was performed as described by Wang H et al. [22]. Briefly, BALB/c mouse (donor) hearts were excised en bloc *via* median sternotomy. Then, the pulmonary artery of the graft was anastomosed end-to-side to the recipient's inferior vena cava, while the ascending aorta of the graft was anastomosed end-to-side to the recipient's abdominal aorta. The heart beating was scrutinized daily by direct abdominal palpation to monitor graft survival. Rejection was determined by complete cessation of cardiac impulses and considered as the ending event. Blood samples were collected from tail veins to measure serum cytokine. Recipient mice were followed up to the date of ending event occurred, or for 100 days if recipient survived for > 100 days.

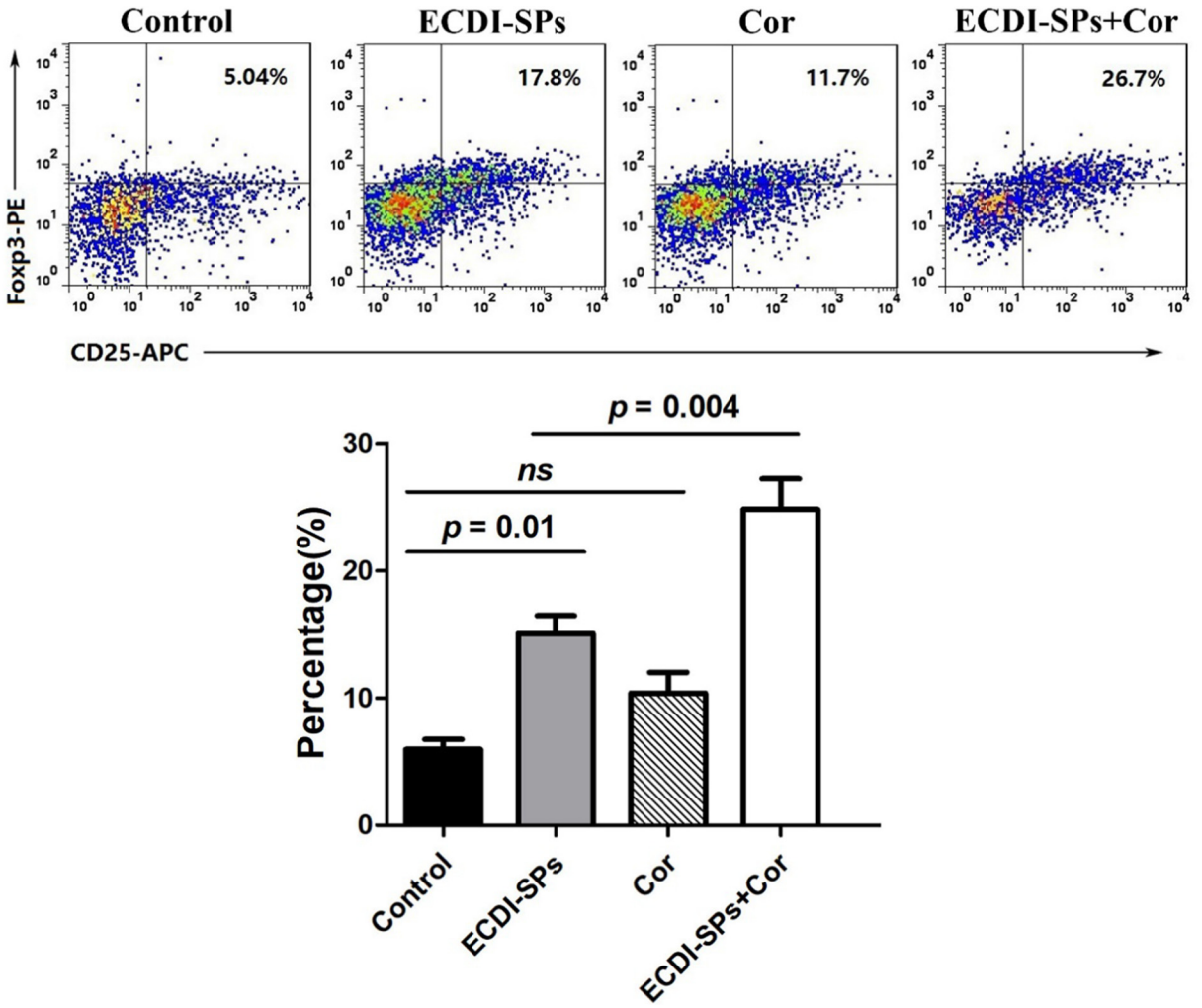

Fig. 2. Cordycepin enhanced the effect of ECDI-SPs on expansion of Tregs. At day 7 after transplantation, splenic monocytes were obtained from 5 to 6 recipients of each group and stained by FITC-conjugated anti-CD4, APC-conjugated anti-CD25, PE-conjugated anti-Foxp3 antibodies.$CD25^+Foxp3^+$ Tregs gated on $CD4^+$ cells were analyzed by flow cytometry. Data were represented as median ± SD and one-way ANOVA was used for analysis. ECDI-SPs, ECDI-treated donor splenocytes; Cor, cordycepin.

2.3. Preparation of ECDI-SPs and cordycepin administration

Spleens harvested from donor (BALB/c) mice were processed into single-cell suspensions. Erythrocytes were removed from suspensions with ACK lysis buffer (BD Biosciences, USA). Then splenocytes (3.2×10^8) were resuspended in 1 ml of PBS containing 150 mg/ml ECDI (Sigma, USA) and incubated on ice for 1 h with gentle agitation. Each recipient was injected with 1×10^8 ECDI-treated splenocytes in 200 μl PBS through penile dorsal vein 7 day before and 1 day after cardiac transplantation. Cordycepin (2 mg/mouse daily; Sigma, USA) was administered intraperitoneally from the operation day to day 7 after transplantation.

2.4. Cytokine detection

Culture supernatants derived from a 4-day mixed lymphocytic reactions (MLR) and recipient mouse serum samples at different time points post-transplantation was collected. Pro-inflammatory cytokines (IL-1β, IL-6, IL-17 and TNF-α) and anti-inflammatory cytokines (IL-10 and TGF-β) were quantified by using ELISA kits (Neobioscience, Shenzhen, China) according to the manufacturer's instructions. Concentrations of each cytokine were calculated from a control standard curve.

2.5. FACS analysis of Tregs

Recipient spleens were obtained on day 7 post-transplantation and processed into single-cell suspensions. After removal of erythrocytes, splenocytes were incubated with FITC-conjugated anti-CD4, APC-conjugated anti-CD25, PE-conjugated anti-Foxp3, and isotype-matched control antibodies (BD Science, Franklin Lakes, USA) according to the manufacturer's instructions. Flow cytometric analysis was performed by using a CaliburBD flow cytometry (BD Science, Franklin Lakes, USA).

2.6. Graft histology and immunohistochemistry

On day 7 after transplantation, recipient mice were sacrificed and cardiac allografts were obtained and harvested into 4% formalin. Cardiac tissue samples were embedded in paraffin and cut into slices with thickness of 4 μm. Hematoxylin and eosin (H&E) staining was used to evaluate graft histology. For immunohistochemistry, specimens were stained for Th17 and Tregs by using biotin-conjugated anti-mouse IL-17 mAb (1:100, rabbit IgG, #sc7927; Santa Cruz Biotech) and anti-mouse Foxp3 mAb (1:100, rabbit IgG, #1054C, R&D), respectively.

2.7. One-way mixed lymphocytic reactions

At day 7 after transplantation, recipient spleens were harvested and

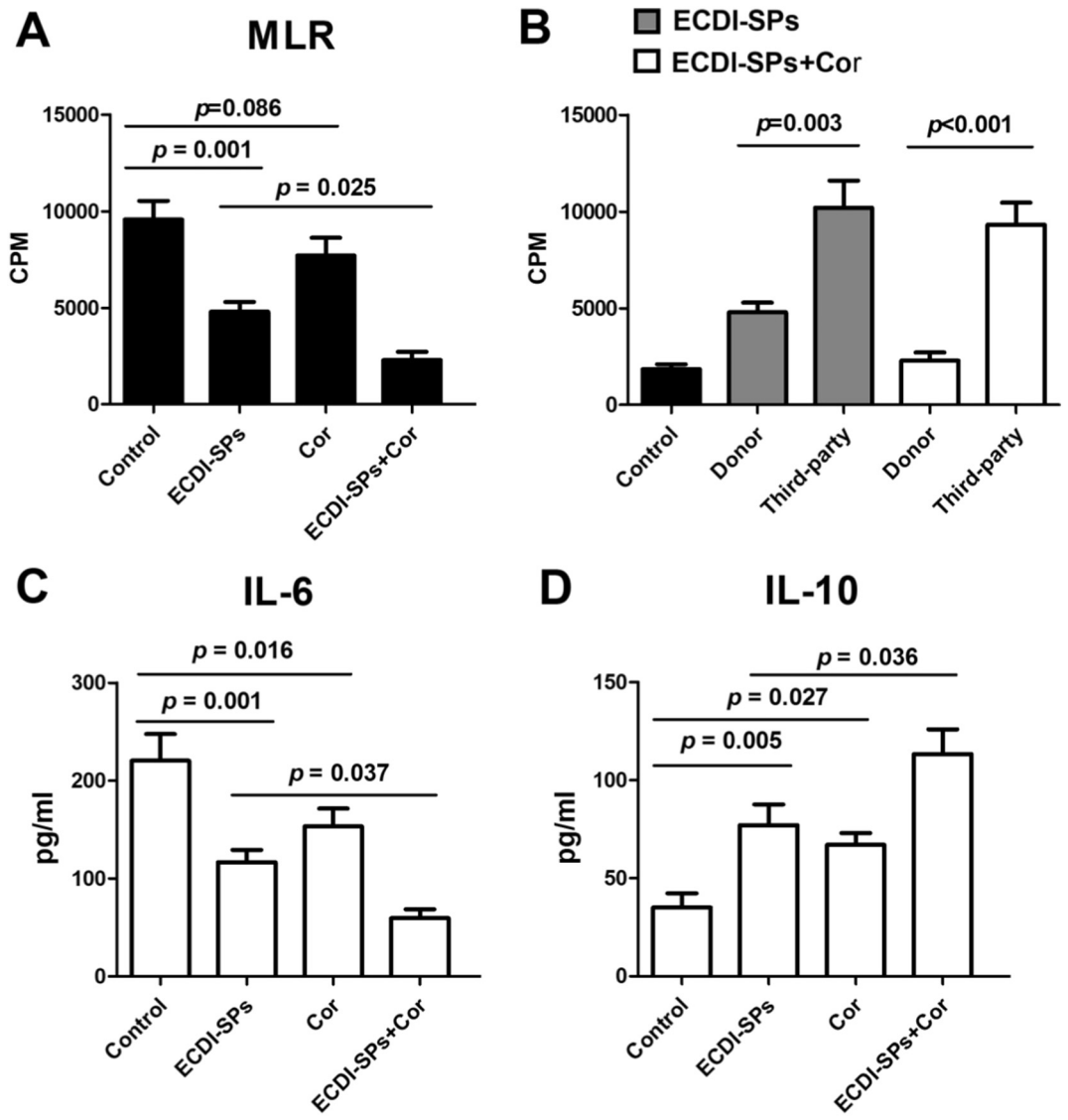

Fig. 3. ECDI-SPs combined with cordycepin altered anti-donor cellular responses and cytokine profile *in vitro*. (A) *In vitro* MLR was set up using mitomycin C-treated BALB/c splenocytes as stimulators and splenic $CD3^+$ T cells from recipients of different groups (on day 7 post-transplant) as responders. Proliferation was measured by ^{3}H-thymidine uptake. (B) T cells from ECDI-SPs-treated mice or ECDI-SPs + Cor-treated mice reacted remarkably less to donor (BALB/c) splenocytes stimulation than third praty (C3H mice). (C) IL-6 and (D) IL-10 concentrations in cultured supernatant were measured by ELISA from different groups. Data were represented as median ± SD from five samples in each group. One-way ANOVA was used for analysis between multiple groups and Student's *t*-test was used for analysis between two groups. ECDI-SPs, ECDI-treated donor splenocytes; Cor, cordycepin.

splenic mononuclear cells were obtained. Splenic $CD3^+$ T cells were isolated by T cell-negative isolation kit (Miltenyi Biotec, Germany) and used as responder cells. Donor (BALB/c) splenocytes were treated with mitomycin C (MMC-SPs) (Sigma, USA) for 30 min at 37 °C and used as stimulator cells. Responder cells were cultured in 96-well plates at a density of 5×10^4 per well and stimulated with 2.5×10^5 stimulator cells. During the last 18 h of 4 days incubation, 1 μCi ^{3}H-thymidine was added into each well. Thereafter, cells were harvested and radioactivity was measured using a scintillation counter (EG&G Wallac, Gaithersburg, MD, USA). Supernatant from parallel cultures were collected for cytokine analysis by ELISA kits.

2.8. Heterotopic cardiac transplantation with prolonged ischemia reperfusion

To evaluate whether cordycepin help to relieve I/R injury, cardiac transplantation with prolonged cold I/R time was performed. Briefly, after removing the donor heart, it was immediately perfused with UW solution through the inferior vena cava and aorta until the vessels of the heart turned clear. Then the donor heart was immersed in UW solution at 4 °C for 8 h, and was implanted into recipient mice as previously described. On day 7 post-transplantation, the recipient mice were sacrificed and the blood was acquired for pro-inflammatory cytokines detection, while the heart grafts were taken for evaluation of pathological score. Morphological assessment of cardiac injury was performed using a semi-quantitative scale of 0 to 4 (4 being the most severe) [23]. The myocardium was assessed by myocytolysis (dissolution of myocytes), inflammatory cells infiltrate, and myocardial necrosis. The degree of injury was measured by the extent of myocardium involved in the above: 1, < 10%; 2, ≥10% and < 30%; 3, ≥30% and < 60%; and 4, ≥60%. Graft survival between groups was also compared after 8 h prolonged I/R.

2.9. Statistical analysis

All statistical analyses were performed using SPSS19.0 software

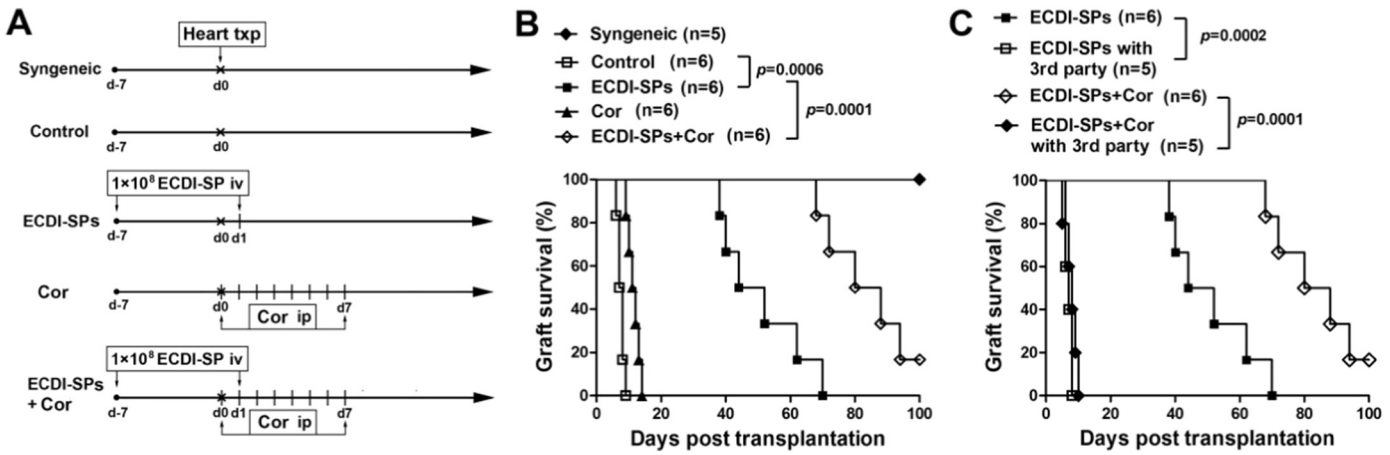

Fig. 4. ECDI-SP combined with cordycepin further prolonged mice cardiac allograft survival in a donor specific fashion. C57B/6 mice (recipients) received BALB/c mice (donor) or C3H mice (3rd party) heterotopic heart transplant and received different treatment. (A) The scheme of treatment for control and different experimental groups. (B) ECDI-SPs monotherapy significantly prolong cardiac allografts survival, and in combination of cordycepin further prolonged mice cardiac allografts survival times. (C) ECDI-SPs or ECDI-SPs + Cor did not protect third party (C3H) mice cardiac allografts. ECDI-SPs, ECDI-treated donor splenocytes; Cor, cordycepin; Tx, transplantation.

(IBM Company, Chicago, IL, USA). Allograft survival among groups was analyzed by Kaplan–Meier, and a log-rank test was used to compare survival difference. The data from cytokine production, flow cytometry, MLR, and immunohistochemistry were represented as mean ± SD and analyzed using one-way ANOVA or Student's *t*-test. A P value $< .05$ was considered statistically significant.

3. Results

3.1. ECDI-SPs combined with cordycepin inhibited pro-inflammatory cytokine production while increased anti-inflammatory cytokine release

The early cytokine milieu is important for ECDI-SPs-induced tolerance [6]. Pro-inflammatory cytokines counter expression and function of negative costimulatory molecules, such as CTLA-4 and PD-L1, which play important roles in ECDI-SPs-induced tolerance [9,24]. To investigate the effect of ECDI-SPs combined with cordycepin on the cytokine expression profiles, serum pro-inflammatory and anti-inflammatory cytokine levels were measured at day 7, 30, and 60 after transplantation. Notably, pro-inflammatory cytokine (including IL-1β, IL-6, IL-17 and TNFα) levels, were significantly reduced by either ECDI-SPs or cordycepin monotherapy, and much more markedly by combined treatment, compared with the control group. In contrast, the anti-inflammatory cytokine levels, such as IL-10 and TGFβ, were significantly increased by ECDI-SPs alone, and much more by combination of ECDI-SPs and cordycepin, whereas cordycepin alone could not increase these anti-inflammatory cytokines. Importantly, recipients treated by combined regimen continued to sustain low levels of pro-inflammatory cytokines but high levels of anti-inflammatory cytokines by day 30 and day 60 (Fig. 1). These results indicated that ECDI-SPs combined with cordycepin exert an additive effect to inhibit pro-inflammatory cytokine release while promote anti-inflammatory cytokine secretion.

3.2. Cordycepin enhanced the effect of ECDI-SPs on expansion of Tregs

To investigate the influence of ECDI-SPs + cordycepin on Tregs, the change of the splenic $CD4^+CD25^+Foxp3^+$ T cells were examined by FACS analysis among different groups at day 7 after transplantation. Compared with the control mice, the percentage of splenic Tregs was significantly increased in recipients receiving ECDI-SPs monotherapy, and much more in those receiving combined treatment. However, cordycepin monotherapy could not change the percentage of Tregs (Fig. 2). These results indicated that cordycepin could enhance the effect of ECDI-SPs on expansion of Tregs.

3.3. Cardiac allograft protection of ECDI-SPs + cordycepin is associated with altered anti-donor cellular responses and cytokine profile

To examine the influence of ECDI-SPs and cordycepin on the response of recipient splenocytes to donor antigens, *in vitro* restimulation by MLRs was set up using mitomycin C-treated donor (BALB/c mice) splenocytes as stimulators and T cells from recipient (C57BL/6 mice) spleens on day 7 after transplantation as responders. T cells from recipients treated with ECDI-SPs or ECDI-SPs + cordycepin showed markedly reduced proliferative responses to donor splenocytes stimulation (Fig. 3A). This hyporesponsiveness of recipient T cells was donor specific, since it was not achieved when third-party stimulator splenocytes were used (Fig. 3B). T cells from cordycepin-treated recipients showed comparable proliferation to donor stimulation as those from control mice. To study the possible hyporesponsiveness mechanisms, cell culture supernatants were collected from each well for cytokine analysis. The results demonstrated that both ECDI-SPs and cordycepin treatment reduced IL-6 levels and elevated IL-10 levels. However, ECDI-SPs combined with cordycepin significantly decreased IL-6 level while profoundly increased IL-10 levels compared with control, ECDI-SPs alone, or cordycepin alone group (Fig. 3C & D). Taken together, these data indicated that enhanced allograft survival observed in the ECDI-SPs + cordycepin may be the result of inhibited T cell proliferation and an altered cytokine profile.

3.4. ECDI-SPs combined with cordycepin induced long-term protection to mouse cardiac allografts

Studies have shown that the production of pro-inflammatory cytokines may hinder ECDI-SPs-inducing tolerance. Thus, suppressing pro-inflammatory cytokines by an anti-inflammatory agent such as cordycepin may enhance graft protection provided by ECDI-SPs. To test this hypothesis, in this study, we used a BALB/c to C57B/6 heart transplant model to confirm the effect of ECDI-SPs combined with a peri-transplant short-course of cordycepin (Fig. 4A). Compared with untreated mice, the survival time of mice receiving ECDI-SPs monotherapy was significantly promoted ($P = .0006$), which is comparable to our previous study [25]. Although cordycepin could prolong mice grafts survival, this protection was transient. However, when combined with ECDI-SPs, this combined regimen further prolonged graft survival

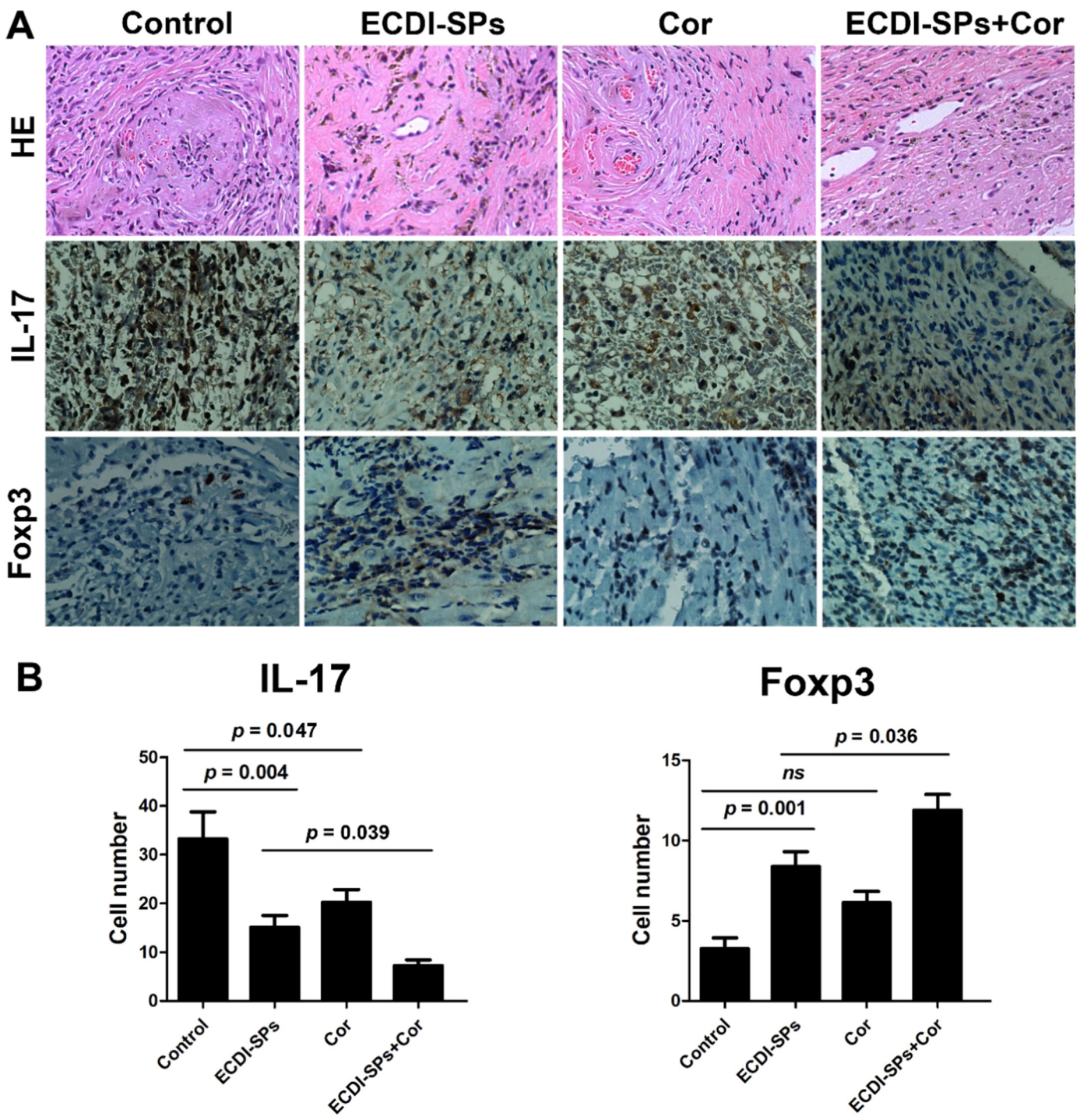

Fig. 5. Combination of ECDI-SPs and cordycepin modulated histologic changes, diminished Th17 infiltration and enhanced Tregs expansion in the allografts. (A) Histological changes of recipient heart allografts in different groups at day 7 after transplantation. Grafts were stained by hematoxylin and eosin (H&E) and by immunohistochemistry using IL-17 and Foxp3 antibodies. (B) Bar graphs show average cell number of infiltrating IL-17$^+$ and Foxp3$^+$ T cells in cardiac allografts which were determined by two different individuals in high-power fields from 12 to 15 different sections from 3 to 4 cardiac grafts of each group. One-way ANOVA was used for analysis between groups. Magnification: ×400. ECDI-SPs, ECDI-treated donor splenocytes; Cor, cordycepin.

compared with ECDI-SPs monotherapy (mean survival time = 86 days, Fig. 4B). Importantly, this protection was donor-specific, since ECDI-SPs alone or ECDI-SPs + cordycepin did not protect C3H mice (third party) cardiac allografts (Fig. 4C).

3.5. ECDI-SPs combined with cordycepin modulate histologic changes, diminished Th17 infiltration whereas enhanced Tregs expansion in the allografts

Histopathologic changes of allograft sections from different groups were assessed by H&E staining at day 7 after transplantation. Acute rejection was witnessed in control group, with typical features characterized by massive mononuclear cell infiltration and intravascular

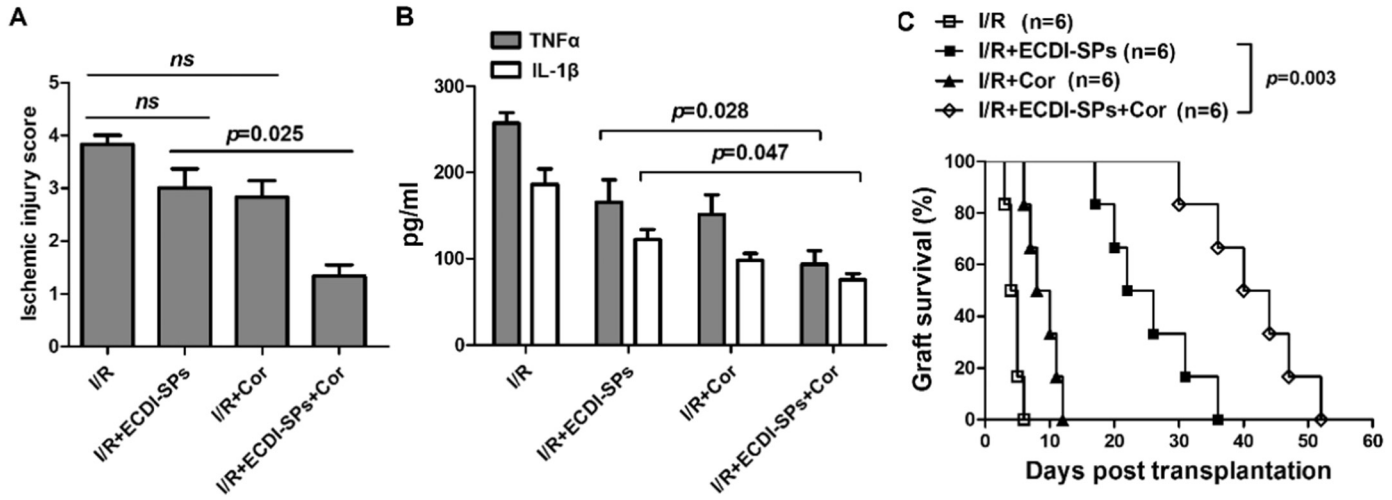

Fig. 6. ECDI-SPs combined with cordycepin protect the heart grafts from I/R injury. (A) The ischemic scores in each group by examining histopathological changes with H&E staining. Expression of TNFα and IL-1β in I/R injured hearts was determined by ELISA. (C) The combination of ECDI-SPs and cordycepin significantly prolong graft survival time in I/R injury hearts.

thrombosis of the cardiac grafts. Cordycepin-treated mice displayed similar histological changes to those in control group. In contrast, ECDI-SPs monotherapy showed less mononuclear cell infiltration without thrombosis. In the combined treatment group, the cardiac tissues were well preserved with minimal cellular infiltration and minimal fibrosis, and had no thrombosis (Fig. 5A).

The different lymphocyte populations infiltrating the cardiac allograft of recipients were examined by immunohistochemistry. ECDI-SPs or cordycepin monotherapy significantly decreased the number of infiltrating Th17 cells, and decreased more dramatically with treatment of ECDI-SPs + cordycepin. In contrast, the presence of Tregs was significantly increased in grafts from ECDI-SPs treated recipients. Cordycepin monotherapy could significantly increase Tregs population. However, when combined with ECDI-SPs, this combined regimen markedly enhanced Tregs presence compared with ECDI-SPs monotherapy (Fig. 5).

3.6. Cordycepin helps to protect the heart grafts from I/R injury and prolong graft survival induced by ECDI-SPs

Donor hearts were excised and preserved with chilled UW solution for 8 h, followed by allograft heterotopic cardiac transplantation. At day 7 after transplantation, the implanted cardiac grafts were harvested to assess I/R injury by examining histopathological changes with H&E staining. The ischemia injury score was significantly lower in the ECDI-SPs + Cor group than in the ECDI-SPs group, indicating that cordycepin provided protection against heart I/R injury (Fig. 6A, $p = .025$). To investigate the change of inflammatory cytokines in I/R injury, we assessed the serum levels of pro-inflammatory cytokines (TNFα and IL-1β) by ELISA. In the ECDI-SPs + Cor group, the serum levels of TNFα and IL-1β were significantly decreased compared with those in the ECDI-SPs group (Fig. 6B). After an 8 h prolonged I/R time, although the graft survival was shorter in each group than those without I/R pretreatment, respectively, ECDI-SPs combined with cordycepin still significantly prolong graft survival compared with ECDI-SPs treated alone in I/R injury model (Fig. 6C).

4. Discussion

ECDI is a water–soluble chemical which has been used as a cross-linker for conjugating peptides to cellular membranes [5], Transfusion of ECDI-fixed donor splenocytes (ECDI-SPs) have been used to induce antigen-specific tolerance in a mouse autoimmune disease model [26], and prolong mouse heart and kidney allograft survival [10,27]. Recent studies demonstrated that transfusion of ECDI-treated donor lymphoid prolonged allogeneic islet graft survival in a nonhuman primate model [28]. The mechanisms of ECDI-SPs appear to regulate T cells by reducing Teff signaling capacity while enhance Treg induction. Since a pre-existing support milieu is indispensable to support Tregs [29], the cytokine milieu is important for the function of ECDI-SPs. As an unavoidable consequence of the organ transplant procedure, ischemia/reperfusion results in the over expression of pro-inflammatory cytokines, such as TNFα, IL-1β and IL-6. These pro-inflammatory cytokines adversely affect graft parenchyma and endothelium and activates graft-destructive immune cell populations [30,31], while inhibiting graft-protective Tregs [32,33]. In contrast, anti-inflammatory cytokines such as IL-10 inhibits T-cell proliferation and pro-inflammatory cytokine release, suppresses the expression of inflammatory inducing chemokine and chemokine receptor genes [34]. Increasing IL-10 levels prolong graft survival in a cardiac transplant model [35], whereas inhibition of IL-10 prevented ECDI-SPs-induced immune tolerance [6]. TGF-β is a potent cytokine that plays an important role in apoptotic cell-induced tolerance. TGF-β promotes naive $CD4^+$ T cells differentiating into $Foxp3^+$ Treg phenotype and prevents naive T cells differentiating into tissue-destructive Th17 or Th1 phenotype [29]. Therefore, inhibiting pro-inflammatory cytokine secretion while concurrently promoting anti-inflammatory cytokine release would create a favorable milieu to enhance the graft-protective function of ECDI-SPs.

Cordycepin is a type of nucleoside analogue which is structurally similar to adenosine. Emerging evidence shows that cordycepin functions by reducing pro-inflammatory cytokine production and enhancing anti-inflammatory cytokine secretion. Cordycepin suppresses levels and activity of pro-inflammatory cytokines and chemokines including NO, PGE2, TNF-α, and IL-1β, whereas elevates levels of anti-inflammatory cytokines such as IL-10, IL-1Ra and TGFβ [15–17]. Recent studies showed that cordycepin exerts potent neuroprotective functions against cerebral ischemia reperfusion injury in mice [18,19], and prevents rat hearts from ischemia/reperfusion injury [20].

Base on the previous data, we hypothesized that with a combined use of cordycepin during peri-transplant period would change the cytokine profile from pro-inflammatory to anti-inflammatory, creating a supportive microenvironment for ECDI-SPs to function in allograft protection. In the present study, by using a mouse cardiac transplant model, we aimed to examine the effect of ECDI-SPs combined with cordycepin and investigated the possible mechanisms. We first investigated the cytokine profiles in recipient serum of ECDI-SPs,

cordycepin, or combined regimen of ECDI-SPs + cordycepin. Our results showed that both ECDI-SPs and cordycepin decreased pro-inflammatory cytokine (including IL-1β, IL-6, IL-17 and TNFα) levels, and increased anti-inflammatory cytokine (including IL-10 and TGFβ) levels. Interestingly, the combined regimen of ECDI-SPs and cordycepin showed more efficacy at decreasing pro-inflammatory cytokines and increasing anti-inflammatory cytokines. In our *in vitro* study of MLR, we found that ECDI-SPs + cordycepin was also more effective at decreasing IL-6 level and increasing IL-10 level in culture supernatants than ECDI-SPs or cordycepin monotherapy. These results suggesting that cordycepin help to sustain a supporting cytokine milieu for ECDI-SPs, both *in vivo* and *in vitro*.

Next, we examined the effect of ECDI-SPs combined with cordycepin on Th17 and Tregs. Th17 is one of the potent inducers of autoimmunity and tissue inflammation [36], while Tregs are critical to the induction and maintenance of immune tolerance [37]. Previous studies revealed that Tregs played an important role in ECDI-SPs-induced tolerance since their impairment blocked tolerance induction in mice islet allografts model [12]. In the present study, we found that the combination of ECDI-SPs and cordycepin had a more significant effect on inhibiting Th17 and promoting Tregs both in the spleens and cardiac allografts. The *in vitro* MLR experiment measurement further proved that the allograft protection of ECDI-SPs + cordycepin was associated with altering anti-donor cellular response. Therefore, cordycepin may help establish a favorable Treg:Teff ratio and enhance the tolerance-inducing effect of ECDI-SPs.

Based on these results above, we assessed the efficacy of ECDI-SPs + cordycepin to promote mice cardiac allograft survival. Cordycepin monotherapy only slightly prolonged mice cardiac allograft survival, while ECDI-SPs monotherapy significantly prolonged the survival of cardiac allograft, with MST of 48 days. The combination treatment of these two agents further prolonged the survival of the cardiac allografts, and the MST reached 86 days. Thus, a short course of cordycepin seems helpful to create a supporting cytokine milieu for the graft-protective functions of ECDI-SPs. Importantly, the protection of ECDI-SPs and combined treatment were donor specific, as a third-party allograft was invariably rejected.

I/R injury is associated with increased primary organ dysfunction and subsequent delayed organ function after organ transplantation, and is also correlated with increased episodes of acute and chronic rejection in the long term [38]. Since cordycepin has been reported to exerts potent protective functions against I/R injury in many animal models [18–20], we aimed to investigate whether cordycepin helps to enhance the allograft protection function of ECDI-SPs. In this study, we found that the ischemic injury and the expression of pro-inflammatory cytokines (TNFα and IL-1β) was significantly impeded by ECDI-SPs + cordycepin treatment in a cold I/R model. Furthermore, the survival time was prolonged with combination of cordycepin compared with ECDI-SPs alone in I/R injury heart grafts. Our data suggested that cordycepin protects against I/R injury and supports a protective circumstance for ECDI-SPs for graft protection.

In summary, we examined the potential of an immunotherapy regimen that combines ECDI-SPs with a short-course of cordycepin injection specifically prolonged the survival times of mice cardiac allografts. Cordycepin help to strengthen the effect of ECDI-SPs in impairing donor-specific responses, altering the cytokine profile, reducing Th17 and increasing Tregs. Consequently, combination of ECDI-SPs and cordycepin creates a favorable graft environment resulting in the long-term survival of the cardiac allograft, which may provide a promising therapeutic strategy for tolerance induction in clinical organ transplantation.

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