ORIGINAL ARTICLE

Ethylene carbodiimide-fixed donor splenocytes combined with α-1 antitrypsin induce indefinite donor-specific protection to mice cardiac allografts

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SUMMARY

Peritransplant infusion of ethylene carbodiimide-fixed donor splenocytes (ECDI-SPs) induces protection of islet and cardiac allografts. However, pro-inflammatory cytokine production during the peritransplantation period may negate the effect of ECDI-SPs. Therefore, we hypothesized that blocking pro-inflammatory cytokine secretion while increasing levels of anti-inflammatory cytokines would enhance the tolerance-induced efficacy of ECDI-SPs. The objective of this study was to determine the effectiveness of using ECDI-SPs combined with a short course of α1-antitrypsin (AAT) for induction of tolerance. Using a mice cardiac transplant model, we demonstrated that ECDI-SPs + AAT effectively induced indefinite mice cardiac allograft protection in a donor-specific fashion. This effect was accompanied by modulation of cytokines through decreasing levels of pro-inflammatory cytokines (including IFN-γ, TNF-α, IL-1β, IL-6, IL-17, and IL-23) and increasing levels of anti-inflammatory cytokines (including IL-10, IL-13, and TGF-β), and by inhibition of effector T cells (Teff) and expansion of regulatory T cells (Tregs). Therefore, we concluded that combined ECDI-SPs and AAT appeared to modulate the expression of cytokines and regulate the Teff:Treg balance to create a support milieu for graft protection. Our strategy of combining ECDI-SPs and AAT provides a promising approach for inducing donor-specific transplant tolerance.

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Introduction

As a therapy for end-stage organ failure, organ transplantation is considered as one of the most significant achievements of 20th century medicine. However, chronic rejection remains a barrier to achieving long-term graft and patient survival. The use of immunosuppressive agents could efficiently reduce rejection, but such agents are usually associated with high risk of opportunistic infections, organ toxicity, malignancies and metabolic disorders [1,2]. Therefore, inducing donor-specific immune tolerance is considered as an ideal solution to both prevent allograft rejection and obviate the need for continuous immunosuppression. Strategies for inducing donor-specific tolerance include induction of mixed hematopoietic chimerism, blockade of costimulatory molecules, depletion of peripheral T cells, and induction or expansion of regulatory T cells (Tregs) [2]. Although these approaches have succeeded in rodent animal models, there are still many obstacles in primates and clinical applications.

Recently, studies have demonstrated that intravenous infusion of donor splenocytes fixed with 1-ethyl-3-(3′-dimethylaminopropyl)-carbodiimide (ECDI) is a powerful and safe method for inducing antigen-specific tolerance *in vivo* [3,4]. Specifically, ECDI-fixed donor splenocytes (ECDI-SPs) have been shown to effectively suppress autoimmune diseases in an experimental autoimmune encephalomyelitis (EAE) model and an autoimmune diabetes model [5,6]. ECDI-SPs have also been shown to induce donor-specific tolerance in a mouse model of islet cell transplantation [7]. Similarly, we have previously demonstrated that intravenous infusion of ECDI-SPs significantly prolonged mouse cardiac allograft survival [8]. The mechanisms of ECDI-SPs induced graft protection involve deletion, anergy and regulation of T cells, such as inhibiting effector T-cell (Teff) activity and stimulating Tregs [3,8,9]. However, the establishment and maintenance of a favorable Teff: Treg balance, which is critical in long-term ECDI-SP-induced allo-tolerance [7], is affected by the microenvironment of the allograft. Pro-inflammatory cytokine release during peritransplantation period can promote naïve $CD4^+$ T-cell differentiation into Teff (including Th1 and Th17 cells), preventing Treg induction, and weakening the efficacy of tolerance induction by ECDI-SPs [3,10]. This can explain why ECDI-SPs alone can only prolong graft survival but not indefinitely induce immune tolerance in mouse cardiac allografts. Thus, creating an appropriate support milieu by inhibiting pro-inflammatory cytokine production while simultaneously enhancing anti-inflammatory cytokine release may help to enhance the effect of ECDI-SP-induced tolerance.

α1-Antitrypsin (AAT) is a key serine protease inhibitor, a member of the serpin family, found in high concentrations in serum [11]. AAT has been found to inhibit various enzymes including neutrophil elastase, cathepsin G, proteinase 3, thrombin, trypsin, and chymotrypsin [12]. Additionally, as an acute-phase reactant, AAT demonstrates potent anti-inflammatory, immunosuppressive, and immunoregulatory properties, which are associated with the inhibition of cytokine production, complement activation, and immune cell infiltration [13]. Recent studies have demonstrated that AAT plays an important role in immune regulation, including transplant tolerance, graft-versus-host disease, and autoimmunity [14]. AAT can inhibit pro-inflammatory cytokine production such as IL-1β, TNF-α, and IL-6, and at the same time prevent the expansion and activation of Teff [15,16]. In addition, AAT is also administrated to prevent allogeneic islet cell and marrow allograft rejection and prolong allograft survival [14,17,19].

We hypothesized that using an anti-inflammatory agent such as AAT during peritransplantation period could create a support milieu by reducing pro-inflammatory cytokines and increasing anti-inflammatory cytokines, so as to enhance tolerance induction by ECDI-SPs. The aim of this study is to verify the hypothesis that combined use of ECDI-SPs and AAT could induce donor-specific tolerance and increase long-term graft survival in a full MHC-mismatched mouse cardiac transplant model.

Materials and methods

Animals

Male BALB/c, C57BL/6 (B6), and C3H mice (8–10 weeks old, weighing 20–25 g) were obtained from the Laboratory Animal Center of Sun Yat-Sen University. All animals were maintained under specific pathogen-free (SPF) conditions and were supplied by the Laboratory Animal Center of Sun Yat-sen University. All animal work was conducted under the institutional guidelines of Guangdong Province and approved by the Use Committee for Animal Care. Additionally, all studies were approved by the Sun Yat-sen University Institute Research Ethics Committee.

Abdominal heterotopic cardiac transplantation

Abdominal heterotopic cardiac transplantation was performed as described previously [18]. Briefly, the heart

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of the donor (BALB/c mouse) was excised en bloc via median sternotomy. Then, the ascending aorta and pulmonary artery of the donor were anastomosed end to side to the recipient's abdominal aorta and inferior vena cava, respectively. Direct abdominal palpation of heart beating was used to assess graft survival. Rejection was determined by loss of palpable cardiac impulses and was considered as the ending event. Blood samples were collected from tail veins to measure serum cytokines. The recipient mice were followed up to the date that the ending event occurred, or for 90 days if recipient survived for more than 90 days.

Preparation of ECDI-SPs and AAT administration

Ethylene carbodiimide-fixed donor splenocytes were prepared as previously described [7]. In brief, spleens from BALB/c mice were processed into single-cell suspensions. Erythrocytes were lysed with ACK lysis buffer (BD Biosciences, Franklin Lakes, NJ, USA) and splenocytes were incubated with ECDI(150 mg/ml per 3.2×10^8 cells; Sigma, St. Louis, MO, USA) on ice for 1 h with agitation followed by washing. A total of 1×10^8 ECDI-treated splenocytes in 200 μl PBS were injected intravenously on day −7 and day 1, with day 0 being the day of heart transplantation. AAT (2 mg/mouse; Sigma) was administered intraperitoneally on days −1, 2, 5 and 8.

Cytokine assay

Blood samples were obtained at different time points post-transplantation. Serum pro-inflammatory cytokines (IFN-γ, TNF-α, IL-1β, IL-6 IL-17, and IL-23) and anti-inflammatory cytokines (IL-10, IL-13, and TGF-β) were measured using cytokine 30-plex antibody bead kit (Life Technologies, Grand Island, NY, USA) using luminex 200TM (Luminex Corporation, Austin, TX, USA) according to the manufacturer's instructions. Serum concentrations of each cytokine were calculated from a control standard curve.

FACS analysis

Recipient splenic $CD3^+$ T cells were isolated on day 7 post-transplantation using microbeads (Miltenyi Biotec, Bergisch Gladbach Germany) according to the manufacturer's instructions and analyzed by flow cytometry. Flow cytometry analysis was performed by FACS (Calibur BD, Franklin Lakes, NJ (New Jersey), USA) using CELL QUEST software (BD Science, Franklin Lakes, NJ (New Jersey), USA). FITC-conjugated anti-CD4, PE-conjugated anti-IL-17, APC-conjugated anti-CD25, PE-conjugated anti-Foxp3, and isotype-matched control antibodies were purchased from BD Science, Franklin Lakes, NJ (New Jersey), USA.

Real-time PCR

Total mRNA of fresh cardiac allograft tissues at different time points was extracted with TRIzol reagent (Invitrogen Carlscad, CA, USA) and transcribed to cDNA using a commercial cDNA synthesis kit (Fermentas Inc, Burlington, Ontario, Canada) according to the manufacturer's instructions. First-strand cDNA synthesis was generated from 500 ng of total RNA. Quantification of target and reference (GAPDH) genes was performed in triplicate on ABI Prism 7300 (Applied Biosystems Inc, Carlscad, CA, USA). The primers used in each reaction were as follows: IFN-γ forward 5′-CGCTACACACT GCATCTTGG-3′ and reverse 5′-TTTCATGTCACC ATCCTTTTGCC-3′; IL-4 forward 5′-TCACTGACGGCA CAGAGCTA-3′ and reverse 5′-TCTGTGGTGTTCTTCGT TGCT-3′; IL-17A forward 5′-ATCTGTGTCTCTGATGCT GTTG-3′ and reverse 5′-ATCAGGGTCTTCATTGCG GT-3′; IL-10 forward 5′-GGTTGCCAAGCCTTATCGGA-3′ and reverse 5′-AATCGATGACAGCGCCTCAG-3′; TGF-β forward 5′-AGGGCTACCATGCCAACTTC-3′ and reverse 5′-CCACGTAGTAGACGATGGGC-3′; Foxp3 forward 5′-CTTAGAGAAGACAGACCCATGCT-3′ and reverse 5′-TCAGAGGCAGGCTGGATAAC-3′; GADPH forward 5′-AGGAGCGAGACCCCACTAACA-3′ and reverse 5′-AGGGGGGCTAAGCAGTTGGT-3′. Following normalization to GAPDH gene, expression levels for target gene were calculated using the comparative threshold cycle method.

One-way mixed lymphocytic reactions

One-way mixed lymphocytic reactions (MLRs) were performed at day 7 after cardiac transplantation. Splenic $CD3^+$ T cells were purified from recipient mice using a T cell-negative isolation kit (Miltenyi Biotec) as responder cells and were labeled with 0.5 μM carboxyfluorescein diacetate succinimidyl ester (CFSE; eBioscience, San Diego, CA, USA) for subsequent assessment of T-cell proliferation. T cell-depleted splenocytes from donor mice were irradiated at 25 Gy and used as stimulator cells. A total of 1×10^5 responder cells were cultured in a round-bottom 96-well plate in RPMI-1640 culture medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 mg/ml streptomycin and stimulated with 5×10^5 stimulator cells.

After culture for 4 days, cells were harvested and T-cell proliferation was quantified by CFSE dilution using flow cytometry (Callibor BD).

Measurement of antidonor antibody responses

Thymocytes were harvested from donor thymus and 5×10^5 donor thymocytes were blocked with 1 μl Fc Blocker (Biolegend, San Diego, CA, USA) followed by incubation with plasma samples from recipients (1:4 dilution) on ice for 1 h. Cells were then washed and stained with PE-labeled anti-mouse IgM antibody (eBiosciences) and APC-labeled anti-mouse IgG antibody (Biolegend), and analyzed by FACS. Negative control was provided by incubation with naïve mouse sera.

Graft histology and immunohistochemistry

At different time points after transplantation, recipient mice were sacrificed and heart allograft sections stained by hematoxylin and eosin (H&E) staining and immunohistochemistry. For immunohistochemistry, cardiac tissues were evaluated for the presence of $CD8^+/IL\text{-}17^+/Foxp3^+$ T cells using biotin-conjugated anti-mouse CD8α mAb (1:100, rat IgG_{2A}, #53-6.7; R&D), anti-mouse Foxp3 mAb (1:100, rabbit IgG, #1054C, R&D), and anti-mouse IL-17 mAb (1:100, rabbit IgG, #sc7927; Santa Cruz Biotech, Dallas, TX (Texas), USA). Intragraft infiltration of $CD8^+/IL\text{-}17^+/Foxp3^+$ T cells was quantified by counting positively stained cells from different slices from 3 to 4 recipients of each group.

Statistical analysis

All statistical analyses were performed using SPSS19.0 software (IBM Company, Chicago, IL, USA). Kaplan–Meier analysis was used for graft survival analysis, and a log-rank test was used to compare survival among groups. Quantitative variables were represented as median ± quartile and analyzed by nonparametric test (rank-sum test) if the sample size was small and the data did not have normal distribution. If quantitative variables had normal distribution, data were represented as mean ± SD, and Student's *t*-test was used for analysis. A P value <0.05 was considered to be statistically significant.

Results

Combined treatment of AAT and ECDI-SPs induced indefinite donor-specific protection to mouse cardiac allografts

In this study, we hypothesized that the combined use of AAT, a proinflammatory inhibitor, may suppress proinflammatory cytokines and enhance graft protection provided by ECDI-SPs. To test this hypothesis, a BALB/c to B6 cardiac transplant model was used (scheme shown in Fig. 1a). Similar to our previous study [8], mice receiving ECDI-SPs alone exhibited prolonged graft survival relative to untreated controls [median survival time (MST) = 42 days for ECDI-SPs-treated mice versus 7 days for control mice, $P = 0.0006$; Fig. 1b], but

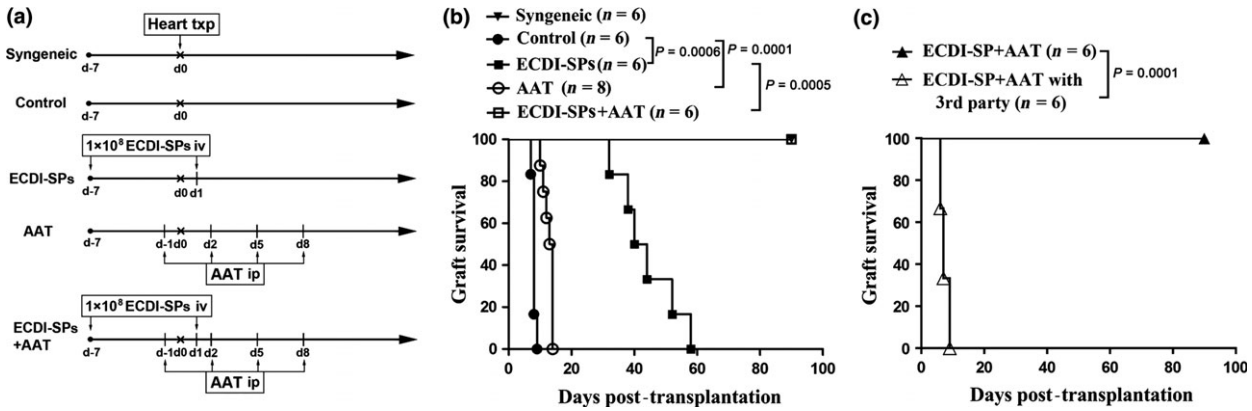

Figure 1 Combined treatment of AAT and ECDI-SPs induced indefinite donor-specific protection to mouse cardiac allografts. Heterotopic heart transplant was performed using Balb/c mice as donors, and C57B/6 mice as recipients. C3H mice were used for third-party donors. (a) Scheme of treatment for different experimental groups. (b) ECDI-SPs or AAT alone significantly prolongs cardiac allograft survival, whereas only combination of ECDI-SPs and AAT induced indefinite protection of cardiac allografts (>90 days). (c) ECDI-SPs + AAT induced allograft protection in a donor-specific fashion. Allografts from Balb/c mice achieved indefinite graft protection, while allografts from C3H mice (third party) were all rejected by day 9. ECDI-SPs, ECDI-treated donor splenocytes; AAT, α-1 antitrypsin; Tx, transplantation. Allograft survival was calculated by Kaplan–Meier analysis.

fail to induce indefinite graft survival. Unlike the graft protection seen in other transplant models such as allogeneic islet transplantation [20], protection of allogeneic cardiac grafts by AAT was transient, as all of the mouse heart allografts were rejected by day 14 (Fig. 1b). However, when ECDI-SPs was combined with a peri-transplant short-term use of AAT, the combination therapy led to long-term graft survival (>90 days) in 100% of the recipients. Importantly, cardiac allografts protection induced by ECDI-SPs + AAT was donor specific, because all cardiac allografts were rejected by day 9 when a third-party donor (C3H mice) was used (Fig. 1c).

ECDI-SPs combined with AAT inhibited pro-inflammatory cytokine and increased anti-inflammatory cytokine production

To investigate whether combination of ECDI-SPs and AAT might suppress inflammatory conditions during the peritransplantation period, serum pro-inflammatory and anti-inflammatory cytokines levels were measured by luminex at days 7, 30, and 90 after transplantation. Notably, the serum level of pro-inflammatory cytokines, including IFN-γ, TNF-α, IL-1β, IL-6, IL-17, and IL-23, was significantly reduced by ECDI-SPs or AAT treatment alone, and much more markedly by combined treatment of ECDI-SPs and AAT, compared with the control group. In contrast, the serum levels of anti-inflammatory cytokines such as IL-10, IL-13 and TGF-β were increased by ECDI-SPs or AAT treatment alone, and more by combination of ECDI-SPs and AAT. Importantly, ECDI-SPs + AAT-treated recipients continued to show sustained low levels of pro-inflammatory cytokines and elevation of anti-inflammatory cytokines at day 30 and day 90 (Fig. 2). These results indicated that combined ECDI-SPs and AAT could enhance the effects on inhibiting pro-inflammatory cytokine release while promoting anti-inflammatory cytokine production.

Cardiac allograft protection is associated with markedly reduced expression of pro-inflammatory cytokines, but enhanced anti-inflammatory cytokine expression among allografts

We next investigated the expression of cytokines among allografts. The results showed that ECDI-SPs alone, AAT alone, and ECDI-SPs + AAT treatment significantly decreased pro-inflammatory cytokine (IFN-γ, IL-6, and IL-17) mRNA levels at day 7, and this phenomenon persisted to days 30 and 90 in ECDI-SPs + AAT-treated recipients. In contrast, anti-inflammatory cytokine mRNA (IL-10 and TGF-β) and Foxp3 mRNA were significantly increased in ECDI-SPs-treated, AAT-treated, and combination-treated groups at day 7, and this phenomenon persisted to longer time points (day 30 and 90) in the combination therapy group (Fig. 3). Therefore, AAT appeared to enhance the effect of ECDI-SPs on regulating the expression of cytokines among allografts to create a suitable cytokine milieu for graft protection.

Allograft protection is associated with inhibition of Th17 and expansion of Tregs

We next tried to determine the influence of ECDI-SPs + AAT on Teff (Th17) and Tregs in recipients, which was examined by analyzing splenic IL-17$^+$ and Foxp3$^+$ T cells among different groups. The results showed that the splenic Th17 population was significantly decreased in ECDI-SPs-treated and AAT-treated recipients, and much more markedly in the combination therapy of ECDI-SPs + AAT group (Fig. 4). In contrast, the population of splenic CD4$^+$Foxp3$^+$Tregs was enhanced in recipients of ECDI-SPs or AAT alone treatment, and much more markedly in those that received combination treatment. These results suggest that AAT could enhance the effect of ECDI-SPs on inhibiting Teff (Th17) while promoting Treg expansion.

ECDI-SPs + AAT significantly diminished graft CD8$^+$T-cell and Th17 infiltration, but enhanced presence of graft Foxp3$^+$ cells

Cardiac allografts from different groups of recipients were examined histologically by H&E staining at days 7 and 30 after transplantation. As shown in Fig. 5a, a dense mononuclear cell infiltrate of the cardiac grafts was observed in control recipients, with less intense infiltration in ECDI-SPs or AAT recipients. In the combined treatment group, the cardiac tissues were well preserved with minimal cellular infiltration and minimal fibrosis.

We further examined the different lymphocyte populations infiltrating the cardiac allograft from groups of recipients that received untreated (control), ECDI-SPs- or AAT-treated alone, or ECDI-SPs + AAT-treated at day 7 and day 30. We noted that ECDI-SPs or AAT alone significantly decreased the number of infiltrating CD8$^+$T cells and Th17 cells at day 7, and decreased

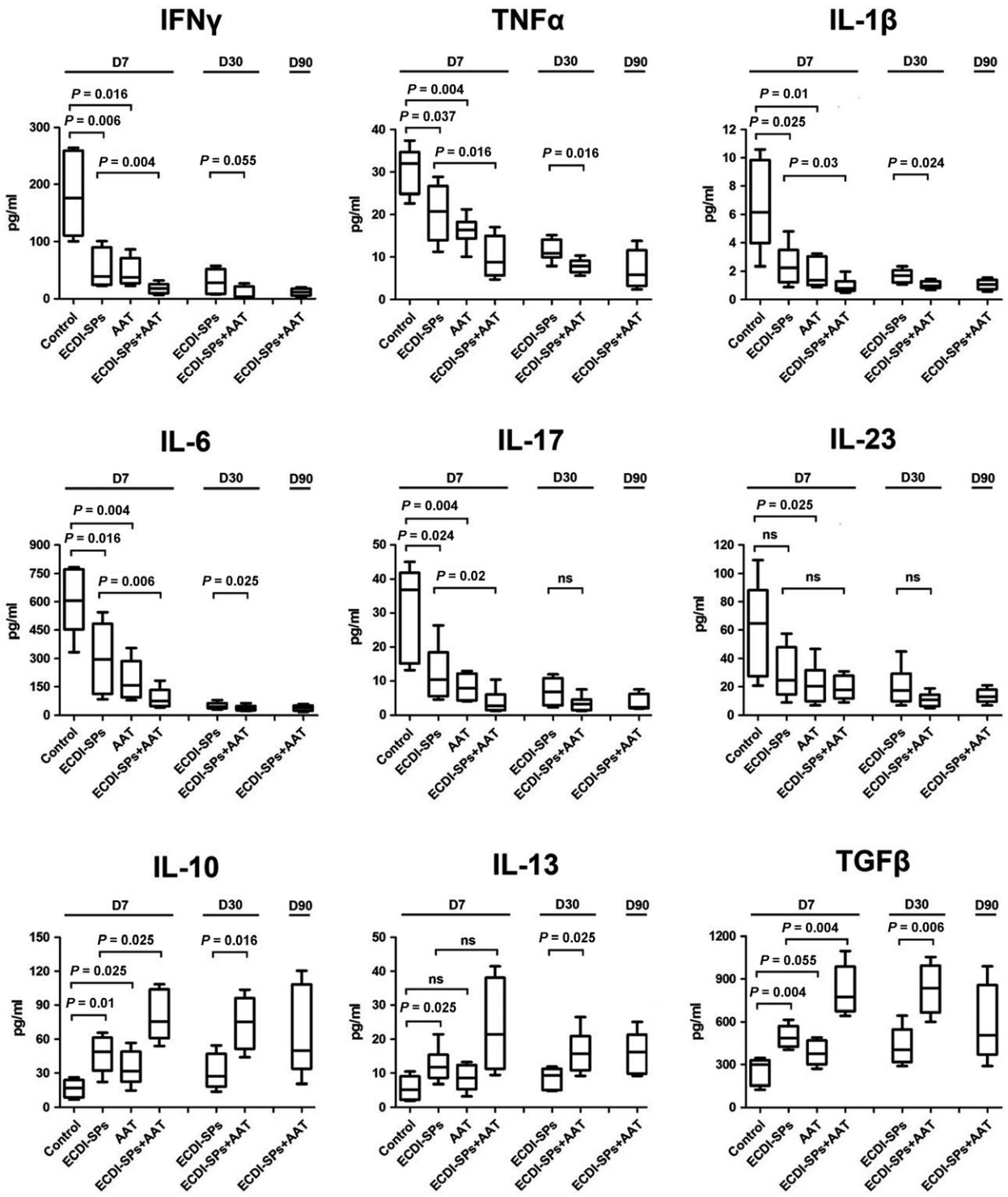

Figure 2 Combined ECDI-SPs and AAT decreased pro-inflammatory cytokines and increased anti-inflammatory cytokines. Serum levels of selected cytokines were measured by luminex at day 7, day 30, and day 90 after transplantation. Data were represented as median ± quartile from serum samples of five or six recipients in each group. Nonparametric test was used for analyzing these data. ECDI-SPs, ECDI-treated donor splenocytes; AAT, α-1 antitrypsin.

more dramatically with treatment of ECDI-SPs + AAT, and this decrease persisted at day 30. In contrast, among the infiltrating lymphocytes at day 7 and day 30, there was an increased presence of Foxp3^{+}Tregs, most significantly in grafts from ECDI-SPs + AAT-treated recipients, and to a lesser extent in grafts from ECDI-

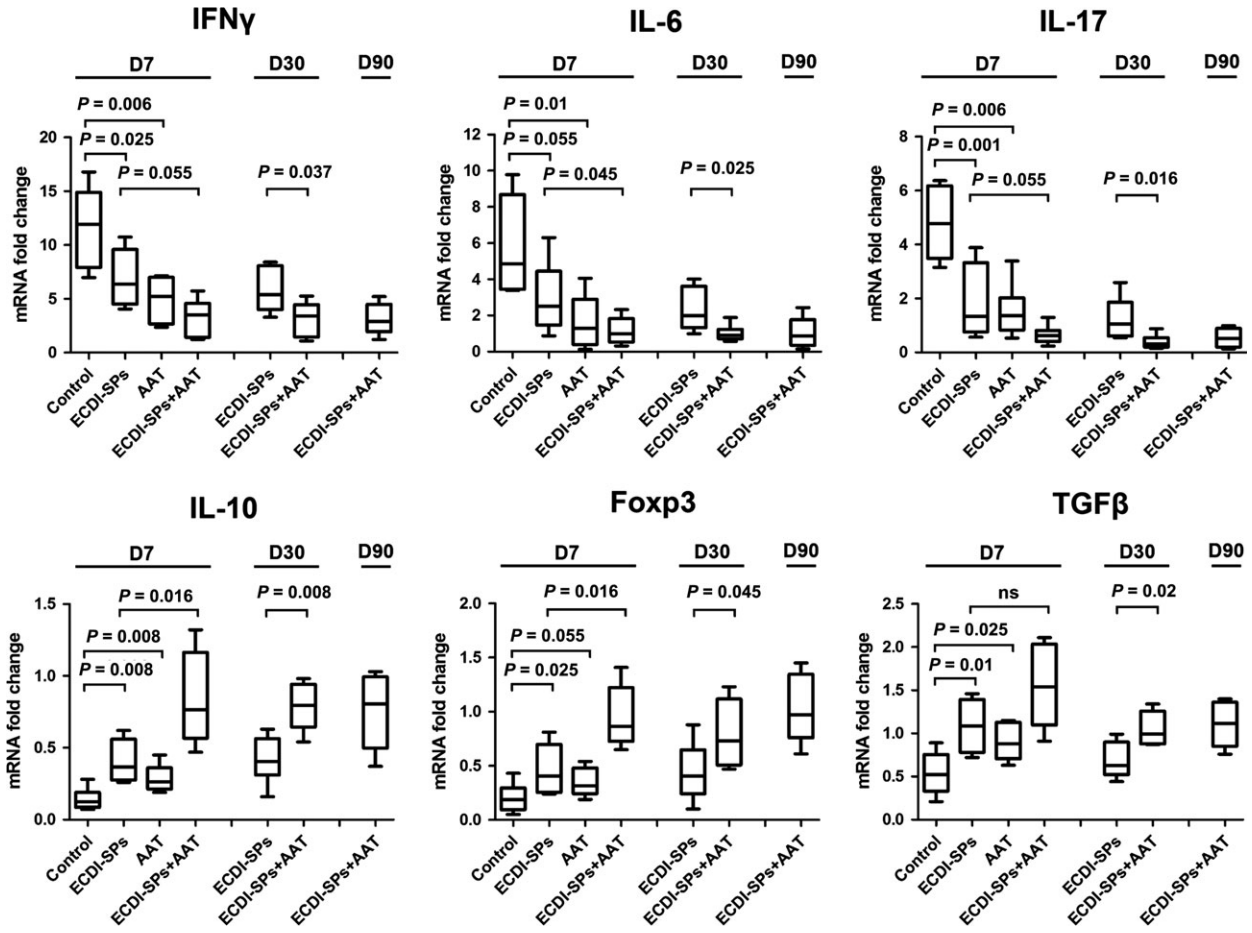

Figure 3 ECDI-SPs + AAT significantly inhibited mRNA expression of pro-inflammatory cytokines and promoted mRNA expression of anti-inflammatory cytokines in cardiac allografts. Quantitative RT-PCR was used to quantify cytokine mRNA expression of heart allografts at day 7 after transplantation. Data were represented as median±quartile from allografts of five or six recipients in each group. Nonparametric test was used for analyzing these data. ECDI-SPs, ECDI-treated donor splenocytes; AAT, α-1 antitrypsin.

SPs-only-treated recipients. However, AAT alone did not increase Foxp3^{+}Treg infiltration compared with the control recipients (Fig. 5).

Cardiac allograft protection is associated with altered antidonor cellular and humoral responses

Antidonor cellular and humoral responses were assessed. *In vitro* restimulation by MLRs was set up using donor (BALB/c mice) splenocytes as stimulators and T cells from recipient (B/6 mice) spleens on post-transplant day 7 as responders. T cells from recipients treated with ECDI-SPs or ECDI-SPs + AAT showed markedly diminished proliferation responses to BALB/c stimulation, whereas T cells from AAT alone-treated recipients showed comparable proliferation to BALB/c stimulation as those from untreated recipients (Fig. 6a,b). Antidonor antibodies (total IgG and IgM) were measured from serum samples acquired at day 14. Serum from naïve mice was used as the negative control. As shown in Fig. 7, untreated controls exhibited elevated antidonor IgG and IgM antibodies as early as day 14 (blue curves). In contrast, ECDI-SPs + AAT-treated recipients showed markedly diminished production of antidonor IgG and IgM at the same time point (red curves).

Discussion

Ethylene carbodiimide-fixed donor splenocytes have been used to induce donor-specific tolerance, which has been described in various models of autoimmune diseases and allograft transplantation. The mechanisms of ECDI-SPs appear to regulate T cells by reducing Teff signaling capacity while enhance Treg induction. It is acceptable that a pre-existing support milieu is indispensable to support Foxp3^{+}Tregs [21], so the cytokine milieu is important for the function of ECDI-SPs. Pro-inflammatory cytokines can inhibit Treg function and activate allo-

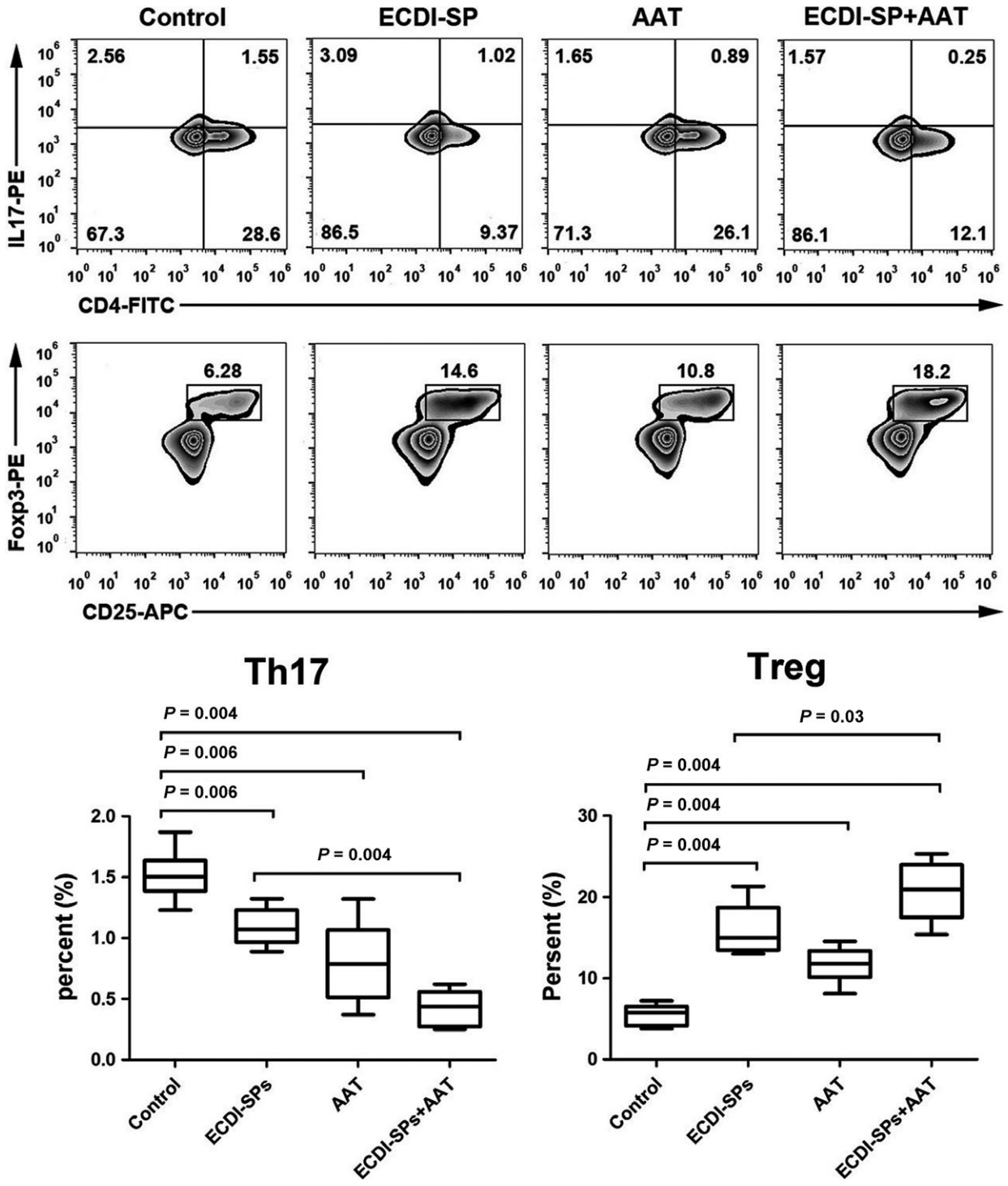

Figure 4 ECDI-SPs combined with AAT significantly inhibited Th17 differentiation and promoted Treg expansion. At day 7 after transplantation, spleens were harvested from 3 to 4 recipients of each group. $CD4^+IL\text{-}17^+$ Th17 and $CD4^+CD25^+Foxp3^+$Tregs were analyzed by flow cytometry. Data were represented as median ± quartile, and nonparametric test was used for analysis. ECDI-SPs, ECDI-treated donor splenocytes; AAT, α-1 antitrypsin.

immune responses. Ischemia/reperfusion is an unavoidable consequence of the organ transplant procedure, which results in a robust release of pro-inflammatory cytokines. This vigorous inflammatory state adversely affects graft parenchyma and endothelium and activates graft-destructive immune cell populations [22–24], while preventing generation of graft-protective Treg cells [25,26]. Various cytokines, including IL-1β, TNF-α, IL-6,

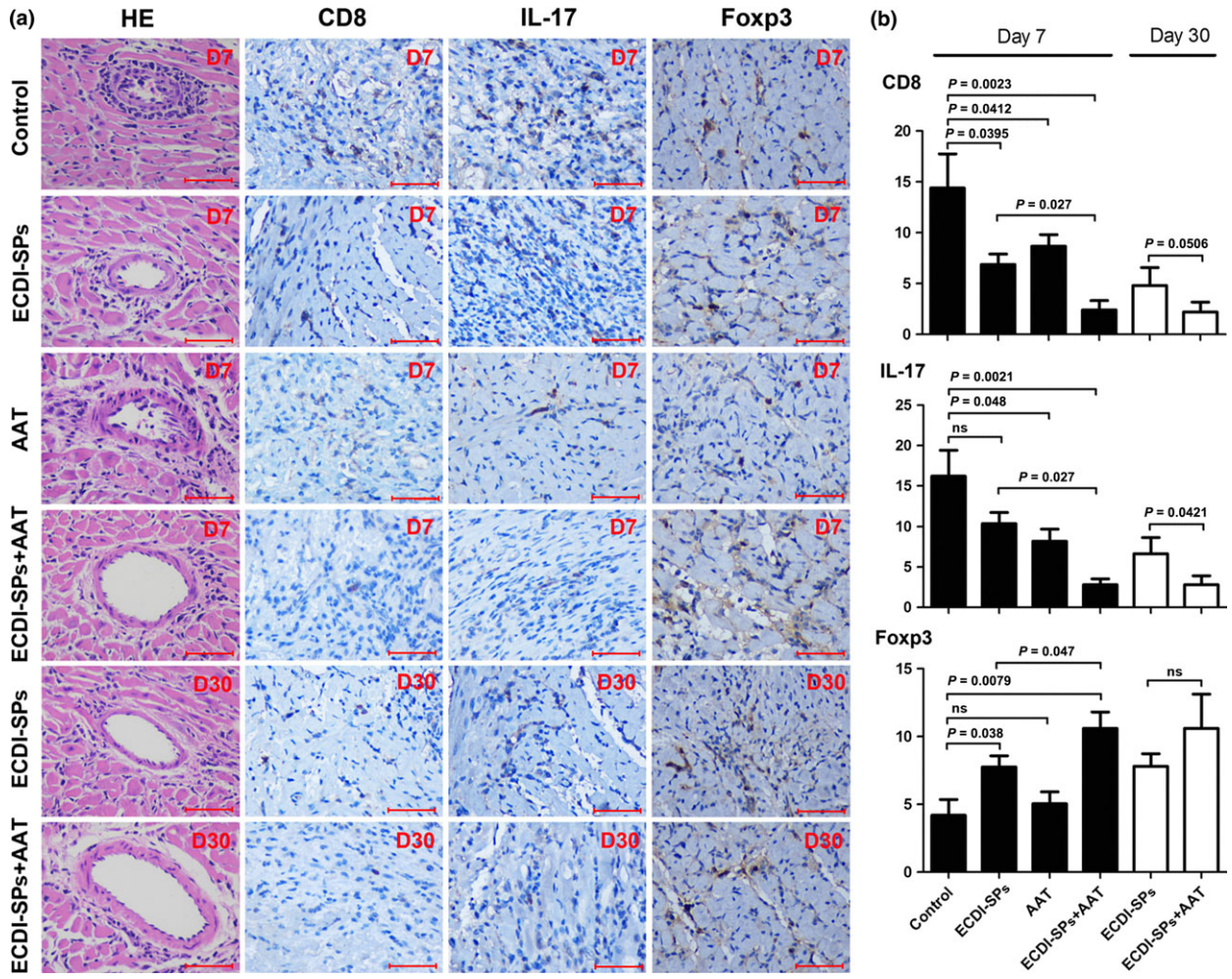

Figure 5 Combined ECDI-SPs and AAT diminished graft infiltration of $CD8^+$ and IL-17^+ cells, but increased $Foxp3^+$ cells. (a) A representative cardiac graft retrieved from control (untreated), ECDI-SPs alone, AAT alone, and ECDI-SPs + AAT-treated recipients at day 7 and day 30. Grafts were stained by hematoxylin and eosin (H&E) and by immunohistochemistry using CD8, IL-17, and Foxp3 antibodies. (b) Bar graphs show average cell numbers per low power field counted by two different individuals from 12 to 15 different sections from 3 to 4 different cardiac grafts from each group. Magnification: ×40. Bar: 50 μm. Data were represented as mean ± SD, and Student's *t*-test was used for analysis. ECDI-SPs, ECDI-treated donor splenocytes; AAT, α-1 antitrypsin.

and IL-21, could counteract Treg suppression of activated Teff [27,28]. For instance, IL-6 plays a pivotal role in directing the immune response toward an inflammatory phenotype and away from a regulatory response. It drives naïve T cells to differentiate into Th17 cells, inhibits the generation of $CD4^+Foxp3^+$ from $CD4^+Foxp3^-$ T cells, and inhibits the suppressive function of natural Tregs [29,30].

In contrast, anti-inflammatory cytokines such as IL-10 induces T-cell anergy by inhibiting proliferation and cytokine production. Recent studies indicate that IL-10 mediates the protective responses from immune-mediated damage that occurs in organ transplantation [31]. This is consistent with the observation that increasing IL-10 levels prolong graft survival in a cardiac transplant model [32,33]. Another anti-inflammatory cytokine TGF-β directs commitment of donor-activated $CD4^+$ T cells into a $Foxp3^+$Treg phenotype and prevents differentiation of T cells into tissue-destructive Th17 or Th1 modes [21].

Thus, blocking pro-inflammatory cytokine release while at the same time increasing anti-inflammatory cytokine production would create a support milieu and enhance the immune regulatory effects of ECDI-SPs. Suppressive agents such as rapamycin could enhance IL-10 production and decrease pro-inflammatory cytokine levels, and prolong allograft survival. However, due to their side effects of the immune inhibitors, there is a need to find safer and more effective agents to inhibit pro-inflammatory cytokine production.

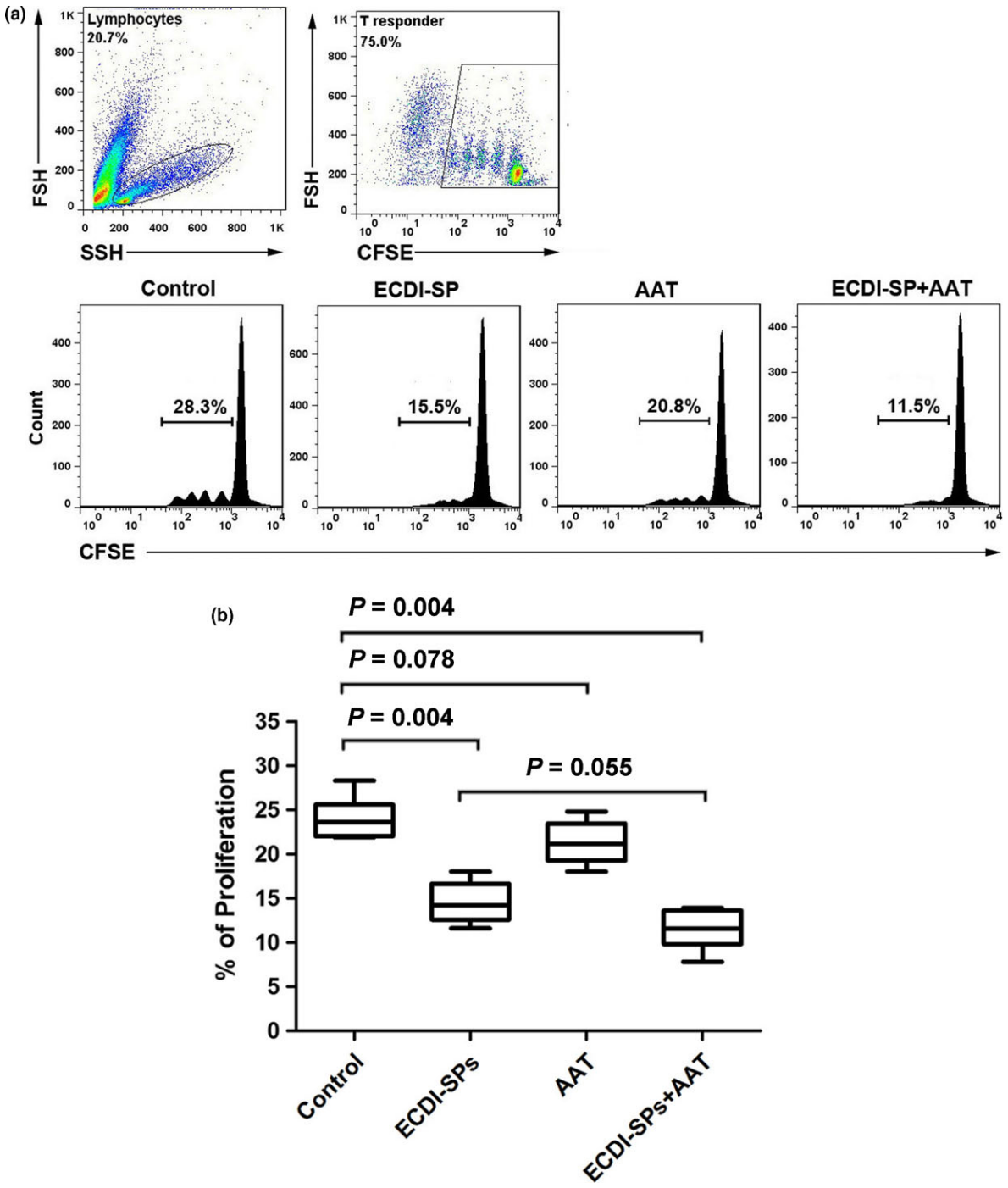

Figure 6 Cardiac allograft protection is associated with altered antidonor cellular responses. *In vitro* restimulation by mixed lymphocytic reactions (MLRs) was set up using BALB/c splenocytes as stimulators and splenic $CD3^+$ T cells from recipients of different treatment groups and controls (on day 7 post-transplant) as responders. (a) Proliferation of T cells was measured by CFSE dilution using flow cytometry. (b) The percentages of responder T-cell proliferation among different groups. Results shown in b were represented with median ± quartile from three independent experiments. Nonparametric test was used for analyzing these data.

α1-Antitrypsin is a serine protease inhibitor present in the serum, which plays important roles in limiting host-tissue injury triggered by neutrophil elastase. Beyond this ability, AAT possesses anti-inflammatory and immune modulatory properties [34]. Emerging evidence shows that AAT functions by reducing pro-inflammatory cytokine production, enhancing anti-inflammatory cytokine secretion, and expansion of

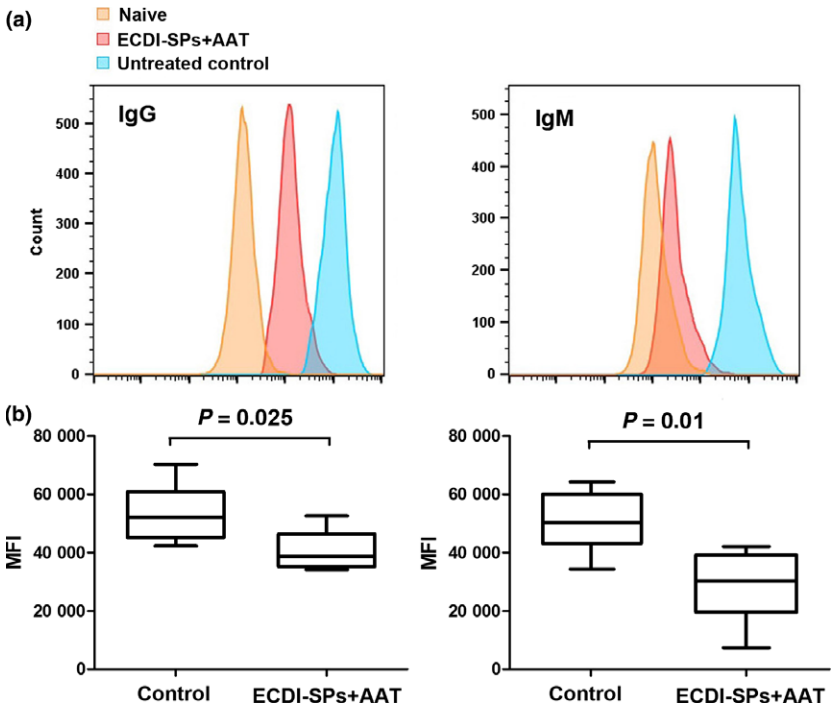

Figure 7 Cardiac allograft protection is associated with altered antidonor humoral responses. (a) Serum total antidonor IgG and IgM were measured on day 14 from recipients treated with ECDI-SPs + AAT or control untreated recipients. (b) Bar graphs show serum MFI of IgG and IgM antibody intensities tested from six mice in each group. MFI, mean fluorescent intensity. Data were represented as median ± quartile, and nonparametric test was used for analysis. ECDI-SPs, ECDI-treated donor splenocytes; AAT, α-1 antitrypsin.

Tregs [35]. The cellular targets of AAT include non-T cells such as dendritic cells [20], B lymphocytes [36,37], macrophages, and neutrophils [38], resulting in reduced levels and activity of inflammatory mediators such as IL-1β, TNF-α, monocyte chemotactic protein (MCP)-1, and nitric oxide, as well as elevating levels of IL-10 and IL-1 receptor antagonist (IL-1Ra) [39,40]. Previous studies demonstrate that AAT monotherapy prolongs survival of islet allografts [17], induces immune tolerance to allografts [20], and prevents development of diabetes in NOD mice [40], shifting the cytokine environment from pro- to anti-inflammatory.

As ischemia/reperfusion is an unavoidable consequence of the organ transplant procedure which leads to over expression of proinflammatory cytokines, we hypothesized that with a combined use of AAT during peritransplantation period could change the balance of pro-inflammatory and anti-inflammatory cytokines and create a supportive microenvironment for ECDI-SPs. Thus, in the present study, we examined the effect of the combination of ECDI-SPs and AAT using a fully MHC-mismatched mouse heart transplant allograft model and investigated the possible mechanisms.

We first assessed the effect of combined ECDI-SPs and AAT treatment on survival in the mouse heart allograft model. Unlike the effect in islet transplantation [20], AAT only transiently prolonged mouse cardiac allograft survival, and the MST only reached 14 days, whereas ECDI-SPs significantly prolonged the survival of heart allograft, and the MST reached 42 days. Why AAT only transiently prolonged allograft survival could be explained by two reasons. Firstly, the half-life period of AAT is short, only 3–5 days, so the function of AAT *in vivo* is transient. Secondly, it has been reported that using hAAT in mice could induce anti-hAAT antibodies in mice [38], which could neutralize the effect of hAAT in the long term. ECDI-SPs can not only inhibit the release of proinflammatory cytokines, but also prolong graft survival through inducing macrophage IL-10 secretion by regulating PD-L1 expression, or inducing Tregs by enzyme indoleamine 2,3 dioxygenase (IDO) expression [6–8]. Only the combination treatment achieved permanent survival (MST >90 days). This effect was donor specific, because a third-party skin allograft was rejected in all recipients. These results indicated that combined ECDI-SPs and AAT could promote indefinite donor-specific protection in the mice cardiac transplant model.

Next, we examined the effect of ECDI-SPs + AAT on the recipient serum cytokine levels and mRNA

expression among allografts. Our results show that ECDI-SPs or AAT, respectively, decreased pro-inflammatory cytokine levels (including IFN-γ, TNF-α, IL-1β, IL-6, IL-17, and IL-23), and increased anti-inflammatory cytokines such as IL-10, IL-13, and TGF-β. Importantly, the combination treatment was more effective at decreasing pro-inflammatory cytokines and increasing inflammatory cytokines. Similarly, the change in mRNA expression of pro-inflammatory cytokines and anti-inflammatory cytokines was in accordance with the serum cytokine levels. Therefore, our results demonstrate that combined ECDI-SPs and AAT was more effective at decreasing pro-inflammatory cytokine production while increasing anti-inflammatory cytokine production, suggesting AAT treatment could help to produce a supporting cytokine milieu for ECDI-SPs.

Based on the observations above, we examined the effect of ECDI-SPs and AAT on Th17 and Tregs. Both ECDI-SPs and AAT independently inhibited Th17 and induced Treg expansion. However, combination of both agents had a more significant effect on promoting Tregs and inhibiting Th17. Therefore, AAT may promote early establishment of a favorable Treg:Teff ratio and enhance tolerance induction by ECDI. The *in vitro* experiment of MLRs and antidonor antibody measurement further proved that the allograft protection of the combination of ECDI-SPs and AAT was associated with altering anti-donor cellular and humoral responses.

In summary, our studies show that donor ECDI-SPs plus a short course of AAT result in indefinite donor-specific cardiac graft protection through inhibiting Teff and enhancing Treg function. The changes in the cytokine milieu by decreasing pro-inflammatory cytokines while increasing anti-inflammatory cytokines might be the consequence of an effect of AAT, which create a supporting milieu for the tolerance induction by ECDI-SPs. Consequently, combination of these two agents creates a favorable graft environment resulting in the indefinite survival of the cardiac allograft. As it may reduce dependence on immunosuppressive agents and induce long-term donor-specific allograft protection, combined ECDI-SPs and AAT may provide a promising therapeutic strategy for tolerance induction in clinical organ transplantation.

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Conflicts of interest

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