Protective effect of trichostatin A on CD19^{+}CD5^{+}CD1d^{high} regulatory B cells in heart transplantation

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Abstract. Heart transplantation is widely used for the treatment of several heart diseases. Regulatory B cells (Breg cells) serve a critical role in immune tolerance. However, the role of Breg cells in immune tolerance in the context of allogeneic heart transplantation remains poorly understood. The present study aimed to explore the effect of histone deacetylase (HDAC) inhibitor trichostatin A (TSA)-regulated Breg on the regulation of immune tolerance in heart transplantation. By constructing anallogeneic heart transplantation mouse model, and performing flow cytometry, reverse transcription-quantitative PCR, western blotting and carboxyfluorescein succinimidyl esterstaining assays, TSA-regulated Breg cells and their effects on immune tolerance in heart transplantation were evaluated. The results demonstrated that TSA increased the frequency of CD19^{+}CD5^{+}CD1d^{high} Breg cells both *in vitro* and *in vivo*. Moreover, TSA treatment increased the frequency of IL-10 and TGF-β-producing CD19^{+}CD5^{+}CD1d^{high} Breg cells, and IL-10 and TGF-β levels *in vitro* and *in vivo*. TSA administration significantly prolonged the survival rate in a heart transplant experiment model. In addition, the IL-10 inhibitor ammonium trichloro(dioxoethylene-o,o')tellurate partially reduced the survival rate and the percentages of CD19^{+}CD5^{+}CD1d^{high} Breg cells in mice receiving heart allografts. In contrast, anti-CD20 treatment significantly decreased the survival rate in these mice. Collectively, the present findings suggested that TSA may induce immune tolerance following heart transplantation by regulating CD19^{+}CD5^{+}CD1d^{high} Breg cells. These results provide a theoretical basis for the prevention of immunological rejection in cardiac transplantation.

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Introduction

Heart disease is a serious condition that threatens human life worldwide (1,2). In 2017, the incidence of heart disease was ~1.8% worldwide (3). Heart failure is a type of heart disease that leads to body weakness, such as severe fatigue, swelling of the hands and legs and difficulty breathing. During heart failure, the blood pumped by the heart fails to meet the requirements of the organs and tissues of the body (4-6). Possible causes of heart failure include coronary artery disease, valvular disease, congenital heart disease, rhythm disorders and cardiomyopathy (7-11). Heart transplantation is the optimal therapeutic choice for the treatment of end-stage heart failure. However, immunological rejection of heart allografts severely affects the survival of patients following transplantation (12-14). Immune tolerance has been recognized as a potential mechanism that prevents immunological rejection (15,16). While previous studies have suggested that transplantation tolerance is can be induced by various stimuli in animal models and human patients, including regulatory T cells, everolimus and suppressors of cytokine signaling 1 (13,14,17), immune rejection remains a major obstacle to the success of heart transplantation (17,18). Golshayan *et al* (17) demonstrated that the donor-specific Treg cells significantly delayed skin graft rejection and promoted donor-specific transplant tolerance in CBA mice. Therefore, understanding the molecular mechanisms of immune tolerance following transplantation would provide an invaluable insight into novel approaches that could be used to prevent immunological rejection of heart allografts.

B cells servean important role in adaptive immune responses by producing antibodies, presenting antigens to T cells and secreting cytokines (19). Regulatory T cells (Tregs) promote peripheral immune tolerance by interacting with other immune cells and secreting anti-inflammatory cytokines, such as effector T cells and TGF-β. Moreover, a subset of B cells that exert a regulatory function and promote immune tolerance have also been identified, and are referred to as regulatory B cells (Bregs) (20). Breg dysfunction is associated with autoimmune diseases, chronic infections, cancer and organ transplant rejection (21-24). A recent study suggested that Bregs could regulate humoral responses that lead to immune rejection and exert a negative immunoregulatory function similar to that of Tregs in the immune system (25). However, the involvement of Bregs in the maintenance of immune tolerance during heart transplantation remains poorly understood.

Histone deacetylases (HDACs) are enzymes that catalyze the removal of acetyl from histones, and serve an important role in immune cells by altering chromatin structure and regulating specific transcription factors (26). HDAC inhibitors have been used as immunomodulatory agents in B-cell-mediated autoimmune diseases, B cell lymphomas and multiple myelomas can be treated by modulating B cell function (27). HDAC-8 inhibitors affected the regulation of reversion-inducing-cysteine-rich protein with kazal motifs and altered the structure of chromosomes associated with tumorigenesis in leukemia (28).

Trichostatin A (TSA) is a HDAC inhibitor with a broad spectrum of epigenetic activities that regulate immune responses *in vitro* and *in vivo*. Krajewski *et al* (29) demonstrated that epigenetic regulation of mast cell activation during immune responses may occur via TSA modulated histone acetylation. TSA is an organic compound that selectively inhibits the canonical class I and II mammalian HDAC families (30). TSA alters gene expression by interfering with HDAC function via influencing histone acetylation levels and accessibility of DNA within chromatin to transcription factors (31). For instance, previous studies have revealed that TSA could inhibit sirtuin 6-mediated deacetylation of synthetic peptide substrates, as well as of HDAC2 and HD2-type HDACs (32,33).

TSA treatment can induce the expansion of $CD4^+CD25^+$ forkhead box P3 $(Foxp3)^+$ Treg populations in CBA/J mice (34). Bhat *et al* (35) reported that HDAC inhibitors could attenuate the antitumor cytotoxicity of γδ T cells, which correlated with enhanced expression of the programmed death-1 in γδ T cells (35). Previous studies have also observed that HDAC inhibitors can also modulate the balance of acetylation and deacetylation of non-histone proteins, and may be responsible for a wide range of immune disorders, including oncogenes is and immuno-inflammatory disorders (36,37).

Trichloro(dioxoethylene-o,o')tellurate (AS101) is a non-toxic immunomodulator that indirectly inhibits the anti-inflammatory cytokine IL-10 (38). Moreover, AS101 inhibits survivin in both B and T cell lymphoma via inhibition of the tumor autocrine IL10/STAT3 signaling pathway (39).

The effect of HDAC inhibitors on B cell function and immune tolerance following allogeneic heart transplantation are yet to be elucidated. Thus, the aim of the present study was to evaluate the effect of HDAC inhibitors on Breg function in a murine model of heart transplantation. The role of $CD19^+CD5^+CD1d^{high}$ Bregs in heart transplantation-induced immune tolerance was specifically examined. The findings of the present study may provide insights into novel therapeutic approaches for the prevention of immunological rejection of heart allografts.

Materials and methods

Mice. A total of 75 male, 7-9-week-old BALB/c (Haplotype-2; $H\text{-}2^d$) and60 C57BL/6 ($H\text{-}2^b$) mice (average weight 23 g) were obtained from the Animal Center of the Second Military Medical University and housed in a sterile facility in microisolator cages under conditions as follows: 18-25˚C temperature, 50-70% relative humidity, a 12 h light/dark cycle, and free access to food and water. All animals received humane care in compliance with the Principles of Laboratory Animal Care and the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (40). All experiments were approved by the Animal Care Committee of Zhejiang Provincial People's Hospital. A total of 12BALB/c mice were randomly assigned to two groups: i) Mice intraperitoneally administered with PBS (control; n=6); and ii) mice given 1.5 mg/kg TSA intraperitoneally (n=6). TSA was administered every other day for 4 weeks until sacrifice.

Heart transplantation and treatment. Heart grafts from the donor C57BL/6 mice were transplanted into the cervical region of BALB/c mice recipients. Cessation of heartbeat was defined as the endpoint for all experiments. Briefly, a 1.5-cm incision was made in the necks of recipient mice on the right side. The external jugular vein was exposed and the branches of the vein to the head were ligated. The head end of the vein was ligated, the proximal end was clipped with a micro vascular clamp and the vein was cut near the head end ligature. The artery was ligated at the bifurcation of the neck and at the external carotid artery. The artery was cut above and below the ligation, and the lumen was washed with heparinized water. The donor and recipient mice were intraperitoneally injected with 60 mg/kg sodium pentobarbital for anesthesia and placed on the operating table. The abdomen was cut, and 1 ml heparin saline was injected intravenously for 5 sec. The ribs were cut along the bilateral anterior iliac crest, the diaphragm was cut longitudinally and the superior atrium was freely ligated. The distal end of the aorta was cut at the aortic bifurcation, and the pulmonary artery was cut at the base of the left and pulmonary bifurcation. The heart was excised free of fat. The recipient mouse was placed on the operation table and its external jugular vein was ligated to the donor heart and pulmonary artery, and the looper was knotted and fixed. The same method was used to fix the recipient common carotid artery and the donor aorta. Following surgery, the mice were injected intraperitoneally with 50 U/g penicillin to minimize infection and inflammation. In total, four mice were excluded from the study, due to cessation of donor heartbeat within a day post-transplantation.

A total of 42 mice were randomly assigned to four groups: i) Recipient mice administered PBS (control; n=10); ii) recipient mice given 1.5 mg/kg TSA intraperitoneally (n=12); iii) TSA-treated recipients intraperitoneally injected with 0.5 mg/kg AS101 (n=10); and iv) TSA-treated recipients intraperitoneally injected with 10 mg/kg anti-CD20 monoclonal antibody (n=10). TSA, AS101 and anti-CD20 treatments were administered every other day post-transplantation until sacrifice. A total of 48 days post-transplantation, the remaining recipient mice were euthanized by cervical dislocation. The survival rate was monitored every 5 days and tissue samples were acquired following sacrifice. Rejection was determined as complete cessation of cardiac contractility and was confirmed by autopsy as a dark red myocardial section, as well as expansion and deformation in the heart cavity.

Reagents and antibodies. Anti-mouse CD3 (cat. no. 561089; PerCP; clone no. 145-2C11), CD19 (cat. no. 561739; PE-Cy7; clone no. 1D3), CD5 (cat. no. 553020; FITC; clone no. 53-7.3), CD1d (cat. no. 562713; PerCP-Cy5.5; clone no. 1B1),

IL-10 (cat. no. 554468; APC; clone no. JES5-16E3), CD4 (cat. no. 553051; APC; clone no. RM4-5), CD25 (cat. no. 553072; FITC; clone no. 7D4) and Foxp3 (cat. no. 560408; PE; clone no. MF23) antibodies, and brefeldin A (cat. no. 347688) were obtained from BD Biosciences. Anti-mouse TGF-β1 (cat. no. IC1835P; PE; clone no. 1D11) antibody was purchased from R&D Systems. Anti-mouse CD20 (clone no. 5D2) was obtained from Genentech, Inc. AS101 (cat. no. 2446; Tocris Bioscience) was dissolved in PBS at a concentration of 50 μg/ml and stored at 4˚C. TSA, phorbol myristate acetate and ionomycin (PIM) were obtained from Sigma-Aldrich; Merck KGaA.

Splenocyte isolation. Cell suspensions were obtained from the spleen using a 100-μm nylon mesh. Lymphocytes were separated from the cell suspensions using Ficoll (GE Healthcare) gradient centrifugation (805 x g; 20 min) at room temperature. After washing with RPMI-1640 (Invitrogen; Thermo Fisher Scientific, Inc.), cells were resuspended in RPMI-1640 medium supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.). The $CD19^+CD5^+CD1d^{high}$ population was identified via flow cytometry and cells were sorted via fluorescence-activated cell sorting (FACS), using a FACSCanto II flow cytometer (BD Biosciences). The FcR was blocked using Fc Receptor Blocker (Abace Biotechnology) for 15 min at 4˚C before staining. A total of $1x10^6$ cells were suspended in PBS supplemented with 0.1% sodium azide (Sigma-Aldrich; Merck KGaA) and 2% FBS in 96-well plates and incubated with the following fluorochrome-tagged antibodies at 4˚C for 30 min: CD3(1:200), CD19 (1:200), CD5 (1:200) and CD1d (1:200). Flow cytometry data was analyzed using FlowJo version 7.6 software (FlowJo, LLC).

Flow cytometry. Blood samples (500 μl per mouse) were collected from recipient mice eyeballs upon sacrifice. Peripheral blood mononuclear cells (PBMCs) were incubated in RPMI-1640 medium with 10 μg/ml brefeldin A at 37˚C overnight. The cells were labeled with fluorochrome-conjugated monoclonal antibodies against CD3 (1:200), CD19 (1:200), CD5 (1:200) and CD1d (1:200) for 30 min at 4˚C, fixed and permeabilized using a Cytofix/Cytoperm kit (BD Biosciences). The cells were washed and permeabilized in Cytofix/Cytoperm buffer for 20 min at 4˚C. The cells were the nstained with anti-IL-10 and TGF-β1 antibodies at 4˚C for 30 min.

To evaluate the effects of TSA on Tregs *in vitro*, PBMCs were isolated and stimulated with 1 μg/ml anti-CD3 monoclonal antibody alone or with 50 nM TSA for 48 h at 37˚C. For $CD4^+CD25^+Foxp3^+$ Treg staining, PBMCs were incubated in RPMI-1640 medium with 10 μg/ml brefeldin A at 37˚C overnight. Cells were stained with anti-CD4 and CD25 antibodies at 4˚C for 30 min. After being fixed and permeabilized using Cytofix/Cytoperm solution, the cells were stained intracellularly with anti-FoxP3 antibody at 4˚C for 30 min. Stained cells were run on a FACSCanto II flow cytometer (BD Biosciences). The frequency of cytokine-expressing cells was analyzed using FlowJo version 7.6 software (FlowJo, LLC).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from Bregsusing MiniBEST universal RNA extraction kit (Takara Bio, Inc.) according to the manufacturer's protocol. cDNA was synthesized by reverse transcription at 37˚C for 60 min. The expression levels of IL-10 and TGF-β1 were assessed using the SYBR PrimeScript RT-PCR kit (Takara Bio, Inc.). The thermocycling conditions used for qPCR were as follow: 50˚C for 2 min, 95˚C for 10 min followed by 30 cycles of 95˚C for 30 sec, 60˚C for 30 sec, and 72˚C for 30 sec and a final extension at 72˚C for 5 min. GAPDH was used as endogenous control. The following primers were used: IL-10 forward, 5'-CAGAGAAGCATGGCCCAGAA-3' and reverse, 5'-GCTCCACTGCCTTGCTCTTA-3'; TGF-β1 forward, 5'-CACTCCCGTGGCTTCTAGTG-3' and reverse, 5'-GGACTGGCGAGCCTTAGTTT-3'; and GAPDH forward, 5'-AGGTCGGTGTGAACGGATTTG-3' and reverse, 5'-TGTAGACCATGTAGTTGAGGTCA-3'. All relative expressions were calculated using the $2^{-\Delta\Delta Cq}$ method and was reported as fold change (41).

Western blot analysis. Bregs were lysed by incubation in RIPA lysis buffer (Beyotime Institute of Biotechnology) containing 1.0 mM phenylmethanesulfonyl fluoride (Sigma-Aldrich; Merck KGaA). Protein concentrations were quantified for all samples using a BCA Protein Assay Kit (Beyotime Institute of Biotechnology). A total of 1.5 μg of protein sample per lane was loaded and separated using SDS-PAGE on 10% gels, then transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Inc.). After blocking with 5% non-fat dry milk for 1 h at room temperature, the membranes were incubated with primary antibodies against HDAC1 (1:1,000; cat. no. 5356; Cell Signaling Technology, Inc.), IL-10 (1:1,000; cat. no. ab34843; Abcam), TGF-β1 (1:500; cat. no. ab92486; Abcam) antibody, GAPDH (1:500; cat. no. ab9485; Abcam) at 4˚C overnight. Secondary antibodies conjugated with horseradish peroxidase (1:7,500; cat. no. 7074; Cell Signaling Technology, Inc.) were incubated at room temperature for 1 h. The internal reference gene GAPDH was used as control. The protein bands were developed using enhanced chemiluminescence solution (cat. no. ECL808-25; Biomiga Inc.). Protein signals were visualized using the E-Gel™ Imager System with E-Gel™ Adaptor (cat. no. 4466613; Thermo Fisher Scientific, Inc.) and quantified using ImageJ software (version 1.46; National Institutes of Health).

Carboxyfluorescein succinimidyl ester (CFSE) staining. FACS-sorted Bregs were resuspended in pre-warmed PBS + 0.1% BSA (Sigma-Aldrich; Merck KGaA) at a final concentration of $1x10^6$ cells/ml. CFSE (Invitrogen; Thermo Fisher Scientific, Inc.) was added at a final concentration of 10 μM and incubated at 37˚C for 10 min. Following incubation, the cells were washed with PBS and treated with 50 nM TSA or 50 ng/ml phorbol myristate acetate + 1 μg/ml ionomycin + 10 μg/ml lipopolysaccharide (LPS; Sigma-Aldrich; Merck KGaA) and incubated at 37˚C for 3 days. LPS + PIM treatment was used as control. CFSE was performed using the Accuri C6 flow cytometer (BD Biosciences). Cell proliferation was analyzed using FlowJo 7.6 software (FlowJo, LLC).

Statistical analysis. Data are presented as the mean ± SD of three independent experiments. Data were analyzed using an unpaired Student's t-test, or one-way ANOVA followed by Tukey's post hoc test. The Kaplan-Meier method and a

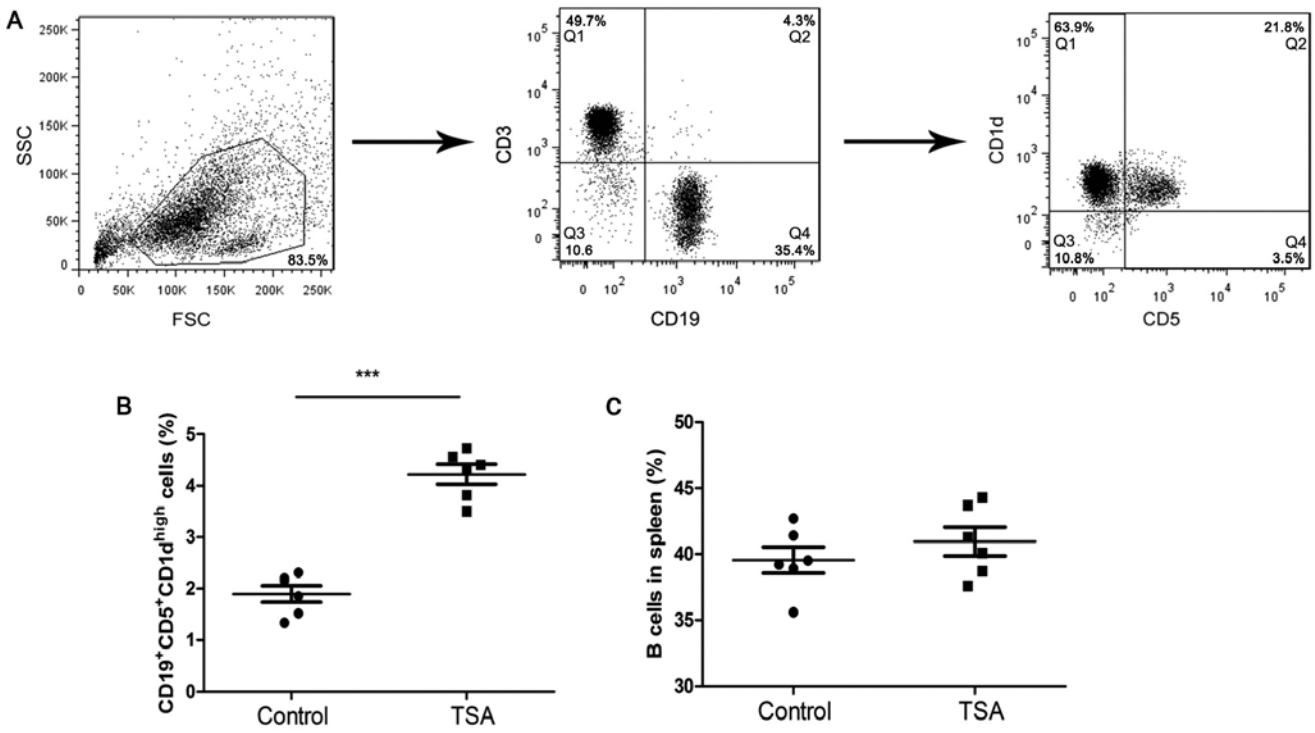

Figure 1. Identification of CD19$^+$CD5$^+$CD1d^{high} Bregs in murine splenocytes. BALB/c mice were treated with TSA intraperitoneally for 4 weeks. (A) Gating strategy for the identification of CD19$^+$CD5$^+$CD1d^{high} Breg cells. Percentages of (B) CD19$^+$CD5$^+$CD1d^{high} Breg cells and (C) CD19$^+$ total B cells were detected following treatment with TSA. Data are presented as the mean ± SD of three independent experiments. ***P<0.001 vs. control. Breg; regulatory B cell; SSC, side scatter; FSC, forward scatter; TSA, trichostatin A.

log-rank test were used to evaluate differences in survival time between different groups. Statistical analysis was carried out using GraphPad Prismversion 5.0 (GraphPad Software, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

TSA promotes CD19$^+$CD5$^+$CD1d^{high} Breg expansion in mice. To investigate the effects of HDAC inhibitors on B cells, mice were treated with TSA for 4 weeks. The gating strategy for the identification of CD19$^+$CD5$^+$CD1d^{high} Bregs is presented in Fig. 1A. TSA significantly increased the frequency of CD19$^+$CD5$^+$CD1d^{high} Bregs, compared with the control group (Fig. 1B). However, the frequency of total B cells in remained unchanged (Fig. 1C).

IL-10-producing CD19$^+$CD5$^+$CD1d^{high} Bregs can promote immune tolerance and prevent immunological rejection (20,21). To determine whether TSA could affect IL1-production by Bregs, the frequency of IL-10-positive CD19$^+$CD5$^+$CD1d^{high} Bregs was analyzed via flow cytometry following treatment (Fig. 2A). TSA significantly increased the proportion of IL-10-producing CD19$^+$CD5$^+$CD1d^{high} Bregs (Fig. 2B). Furthermore, the expression of TGF-β1 was also assessed. Similar to IL-10, the frequency of TGF-β-producing CD19$^+$CD5$^+$CD1d^{high} Bregs increased significantly following treatment with TSA, compared with the control group (Fig. 2C).

IL-10 mRNA expression was increased nearly 4-fold following TSA treatment, in comparison with the control group (Fig. 2D). The relative expression levels of TGF-β1 also demonstrated a significant increase following TSA treatment (Fig. 2E).

TSA activates CD19$^+$CD5$^+$CD1d^{high} Bregs in vitro. In order to investigate the effect of TSA on the proliferation of CD19$^+$CD5$^+$CD1d^{high} Bregs *in vitro*, CD19$^+$ B cells were sorted from total splenocytes via FACS. Relative to controls, treatment with 50 nM TSA significantly increased the proportion of CD19$^+$CD5$^+$CD1d^{high} Bregs *in vitro*. This effect was further enhanced by combined treatment of TSA, LPS and PIM, compared with TSA alone (Figs. 3A and S1). Moreover, the frequency of IL-10 and TGF-β-expressing CD19$^+$CD5$^+$CD1d^{high} Bregs were increased following treatment with TSA, compared with controls. Similarly, treatment with TSA, LPS and PIM had a greater effect compared with TSA alone (Figs. 3B and C and S2). TSA also promoted the proliferation of Bregs, compared with the control group. The combination of TSA, LPS and PIM had a greater effect on Breg proliferation compared with TSA treatment alone (Fig. 3D).

The protein expression levels of IL-10 and TGF-β1 were notably increased following TSA treatment. It was also identified that IL-10 and TGF-β1 were significantly up regulated following combined treatment of LPS and PIM. In addition, HDAC1 expression was significantly reduced following TSA treatment (Fig. 3E and F).

In order to evaluate the effects of TSA on IL-10 expression in T cells, PBMCs were isolated and stimulated with 1 μg/ml anti-CD3 monoclonal antibody alone or with 50 nM TSA. The gating strategy used to identify Foxp3$^+$ Tregs is shown in Fig. S3. TSA treatment, alone or combined with anti-CD3 crosslinking, did not affect the frequency of IL10-positive

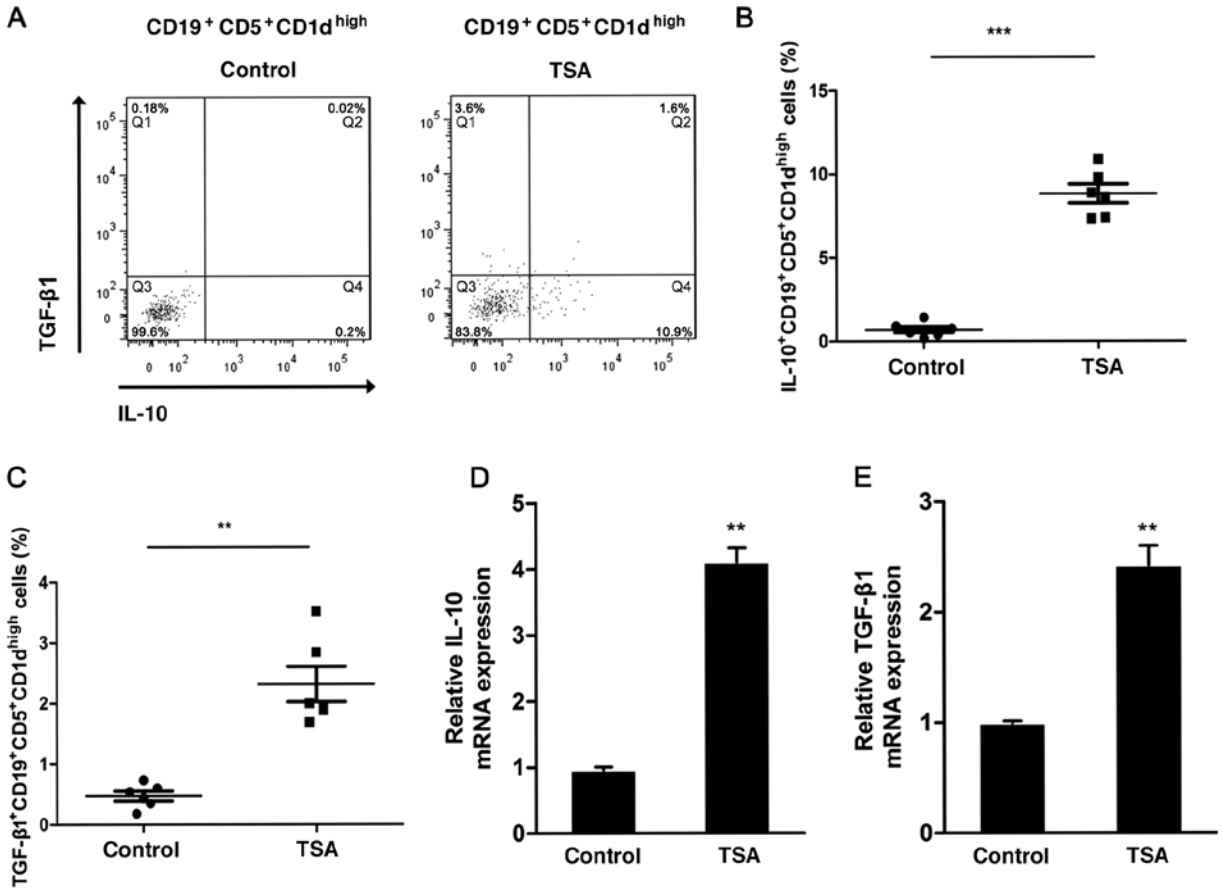

Figure 2. TSA increases the frequency of IL-10 and TGF-β1-producing CD19$^+$CD5$^+$CD1d^{high} Breg cells *in vivo*. (A) Representative dot plots of IL-10$^+$CD19$^+$CD5$^+$CD1d^{high} and TGF-β1$^+$CD19$^+$CD5$^+$CD1d^{high} Breg cells. (B and C) Frequency of IL-10 and TGF-β1-positive cells in CD19$^+$CD5$^+$CD1d^{high} Breg cells. Expression levels of (D) IL-10 and (E) TGF-β1 were detected in the spleen following TSA treatment. **P<0.01, ***P<0.001 vs. control. Breg, regulatory B cell; TSA, trichostatin A.

CD4$^+$ T cells and Foxp3$^+$ Tregs, compared with untreated cells (Figs. 3G and H, S4 and S5). Collectively, these observations suggested that the effect of TSA on IL-10 expression was specific to the Breg subset.

TSA increases the survival rate following heart transplantation in mice. To further examine the effects of TSA-treated Bregs on allograft rejection *in vivo*, a mouse model of heart transplantation was established. Recipient mice were treated with an IL10-inhibitor (AS101) or a B cell-depleting antibody (anti-CD20), as illustrated in Fig. 4A. The effect of the anti-CD20 antibody on B cell numbers was first assessed via flow cytometry, and it was found that anti-CD20 and TSA treatment reduced proportion of B cells to <5% of splenocytes compared with control (Fig. 4B). The effect of TSA on survival rates were also recorded. TSA significantly improved the survival rate of transplanted mice. By contrast, mice co-treated with AS101 had reduced survival rates compared with TSA-treated mice. Similarly, B cell depletion also led to a reduction in survival rates compared with TSA alone. Thus, the protective effects of TSA on heart allografts appear to be mediated by B cells.

PBMCs isolated from the transplant recipients were stimulated with PIM. The frequency of CD19$^+$CD5$^+$CD1d^{high} Bregs was significantly increased in PBMCs from mice treated with TSA compared with control mice. The combination of AS101 and TSA demonstrated no effect on the proportion of CD19$^+$CD5$^+$CD1d^{high} Bregs compared with TSA treatment alone. However, anti-CD20 and TSA administration significantly decreased the percentage of CD19$^+$CD5$^+$CD1d^{high} Bregs, compared with TSA only group (Figs. 4D and S6). In addition, the frequency of IL-10-expressing cells was examined in B cells, Bregs, CD4$^+$ T cells and Foxp3$^+$ Tregs. TSA significantly increased the proportion of IL-10-producing CD19$^+$CD5$^+$CD1d^{high} Bregs and CD19$^+$ B cells, compared with controls. However, the combined administration of AS101 and TSA significantly decreased the proportion of IL-10-producing cells, compared with TSA treatment alone (Figs. 4E and F, S7 and S8). Moreover, TSA, alone or combined with anti-CD20, had no effect on IL-10 production in CD4$^+$ T cells and Foxp3$^+$ Tregs (Figs. 4G and H, S9 and S10).

Discussion

The applicability of allogeneic transplantation for the treatment of heart disease is restricted, partly due to immunological rejection. Currently, there are no effective methods that can induce immune tolerance of the transplanted heart (42,43). Several therapeutic options can reduce immunological rejection following heart transplantation, such as the use of mesenchymal stem cells, small hairpin RNA targeting of

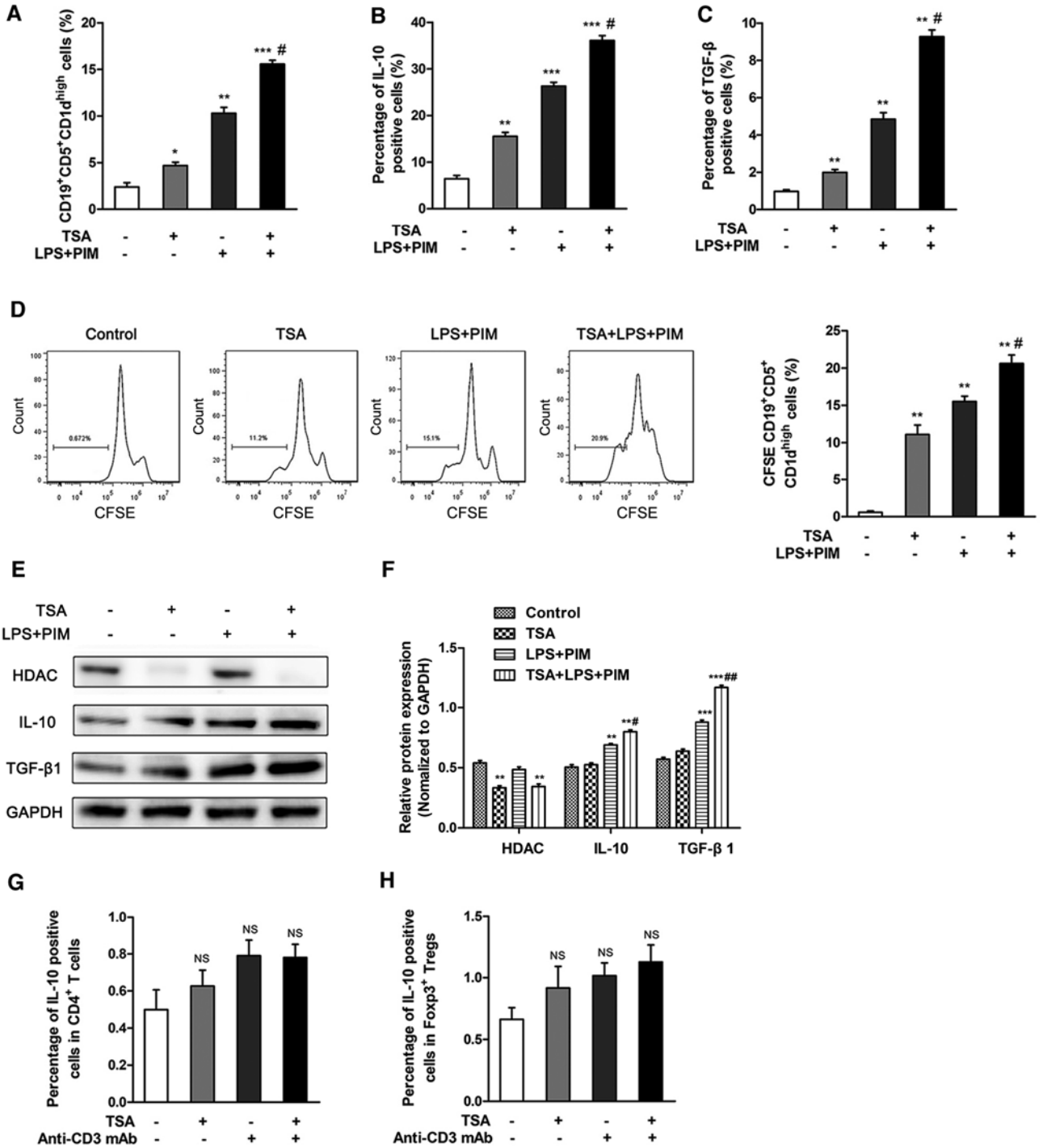

Figure 3. TSA promotes $CD19^+CD5^+CD1d^{high}$ Breg cell proliferation *in vitro*. $CD19^+$ B cells were isolated by fluorescence-activated cell sorting, then stimulated with LPS+PIM and/or TSA for 48 h. (A) Frequencies of $CD19^+CD5^+CD1d^{high}$ Breg cells was detected following treatment. Flow cytometry was used to detect the frequency of (B) IL-10 and (C) TGF-β1-producing Breg cells. (D) $CD19^+CD5^+CD1d^{high}$ Breg cell proliferation was quantified using CFSE staining. (E) Protein expression levels and (F) quantification of HDAC1, IL-10 and TGF-β1. Peripheral blood mononuclear cells were isolated and stimulated with anti-CD3 mAb and/or TSA for 48 h *in vitro*. Flow cytometry was used to detect the positive cells proportion of IL-10 in (G) $CD4^+$ T cells and in (H) $Foxp3^+$ Tregs. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs. control; $^{\#}P<0.05$, $^{\#\#}P<0.01$ vs. TSA. Breg, regulatory B cell; Treg, regulatory T cell; TSA, trichostatin A; Foxp3; Forkhead box protein p3; PIM, phorbol myristate acetate and ionomycin; CFSE, carboxyfluorescein succinimidyl ester; HDAC, histone deacetylase inhibitor; LPS, lipolysaccharide; mAb, monoclonal antibody; NS, not significant.

CD80, CD86 or Toll-like receptors in dendritic cells, immunosuppressive drugs, immune checkpoint inhibitors and total lymphoid irradiation (44-48). However, the clinical benefit of these treatment strategies remains unknown and serious adverse effects often develop. The adverse effects of immunosuppressive regimens with calcineurin inhibitors are linked to increased morbidity and limit the long-term survival of heart transplant recipients (49). Therefore, the investigation of novel therapeutic methods for immune tolerance is urgently required, which depends on an increased understanding of the mechanisms occurring during immunological rejection of a transplanted heart.

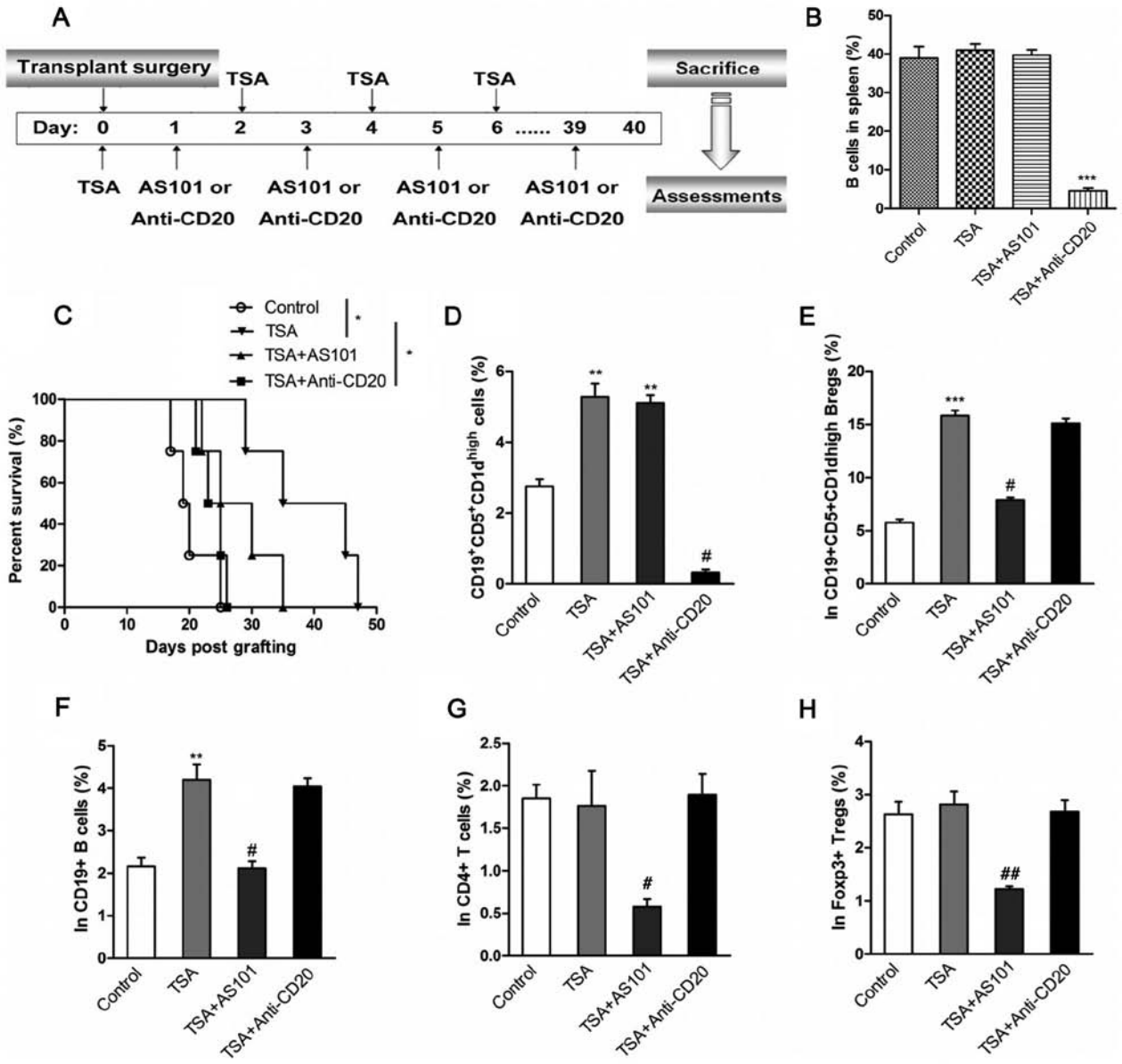

Figure 4. TSA prolongs survival in a heart transplant experiment model. Recipient mice received TSA alone, or combined with AS101 or anti-CD20 monoclonal antibody following heart transplantation. N=8 in each group. (A) Schematic representation of the experimental procedure. (B) Splenic B cell frequency. ***P<0.001 vs. control. (C) Survival rates in each group are presented as a Kaplan-Meier curve. *P<0.05. (D) Frequency of $CD19^{+}CD5^{+}CD1d^{high}$ Breg cells among total B cells. **P<0.01 vs. control; $^{\#}$P<0.01 vs. TSA. Frequency of IL-10-positive cells in (E) $CD19^{+}CD5^{+}CD1d^{high}$ Bregs and in (F) $CD19^{+}$ B cells. **P<0.01, vs. control; $^{\#}$P<0.01 vs. TSA. Frequency of IL-10-positive cells in (G) $CD4^{+}$ T cells and in (H) $Foxp3^{+}$ Tregs, $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. TSA. TSA, trichostatin A; Breg, regulatory B cell; AS101, ammonium trichloro(dioxoethylene-o,o')tellurate.

B cell responses serve a critical role in immunological rejection of transplanted organs (50). Dijke *et al* (51) demonstrated that B cells could regulate cellular immunity, contribute to the genesis of tolerance and induce accommodation. Several studies have reported that HDAC inhibitors could regulate the B cell function in various immunological disorders (27,52,53). For instance, HDAC inhibitors could be used as immunomodulatory agents in order to regulate B cell responses to allogeneic transplantation (54). However, despite the prominent regulatory role of HDACs in the immune system, little is known regarding their function in the context of heart transplantation. In the present study, the regulatory mechanisms of the HDAC inhibitor TSA were investigated in $CD19^{+}CD5^{+}CD1d^{high}$ Bregs, together with its effects on heart transplantation. In heart-transplanted mice, TSA significantly increased the survival rate and the percentage of $CD19^{+}CD5^{+}CD1d^{high}$ Bregs. By contrast, antibody-mediated B cell depletion significantly decreased the survival rate. These results suggested that HDAC inhibitors may serve an essential role in immune tolerance by promoting the expansion of $CD19^{+}CD5^{+}CD1d^{high}$ Bregs.

The HDAC inhibitor sodium valproate has been revealed to increase the expression levels of specific immunosuppressive cytokines, including IL-10 and TGF-β1, in human systemic lupus erythematosus (55). This, in turn, promotes immune tolerance via the alternative activation of monocyte-derived macrophages in patients with systemic lupus erythematosus (55). Consistent with this previous study, an increase in the expression levels of IL-10 and TGF-β1 was observed in the present study in TSA-treated mice. Moreover, the proportion of IL-10 and TGF-β-producing $CD19^{+}CD5^{+}CD1d^{high}$ Bregs

was increased following TSA treatment. Thus, TSA could increase the levels of the immunosuppressive cytokines IL-10 and TGF-β1 in Bregs. Similarly, TSA increased the proportion of $CD19^+CD5^+CD1d^{high}$ Bregs, as well as that of IL-10 and TGF-β-positive Bregs *in vitro*. This effect was enhanced following the combined treatment of LPS and PIM, suggesting TSA could increase LPS- and PIM-induced Bregs. However, the frequency of IL-10-positive cells remained unchanged in in $CD4^+$ T and $Foxp3^+$ Tregs.

The IL-10 inhibitor AS101 partially reduced the frequency of Bregs, including IL-10-producing Bregs in TSA-treated, heart-transplanted mice, suggesting that TSA stimulation may promote immune tolerance by enhancing IL-10 expression in Bregs. Furthermore, in the *in vivo* heart transplant model, AS101 reduced the survival rate and the percentage of $CD19^+CD5^+CD1d^{high}$ Bregs. Similarly, B cell depletion significantly decreased the survival rate, suggesting that TSA-mediated $CD19^+CD5^+CD1d^{high}$ Bregs may promote immune tolerance by enhancing IL-10 expression. Although the protective effects of TSA on heart transplant survival were demonstrated to involve IL-10 and TGF-β1 expression in Bregs, the molecular basis of TSA function requires further investigation.

In conclusion, TSA administration significantly prolonged the allograft survival in a heart transplant model. The present study demonstrated that HDAC inhibitors can promote immune by regulating the expansion of Bregs and promoting the secretion of immunosuppressive cytokines. The present findings provided a potential therapeutic strategy for the prevention of immunological rejection in cardiac transplantation.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

BZ and YC conceived the project. BZ, FM and CW performed the experiments. HX and ZL analyzed and interpreted the data. BZ wrote the article and YC revised the article. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All experiments were approved by the Animal Care Committee of Zhejiang Provincial People's Hospital.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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