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ORIGINAL ARTICLE

Retinoic acid attenuates acute heart rejection by increasing regulatory T cell and repressing differentiation of Th17 cell in the presence of TGF-β

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Summary

Retinoic acid (RA), in a transforming growth factor beta (TGF-β)-dependent manner, promotes differentiation of regulatory T cells (Tregs) but inhibits the differentiation of Th17 cells *in vitro* from naive $CD4^+$T cells. In addition, transfer of induced Tregs (iTregs) reduces rejection. We therefore examined whether RA could attenuate acute cardiac transplant rejection *in vivo* in a mouse model by regulating the reciprocal differentiation of Tregs and Th17 cells. The iTregs and naive T cells were respectively transferred into congenic mice. Two weeks later, the percentages of transferred cells and Forkhead box P3 (FoxP3)+ Tregs were measured in spleen. Mice with cardiac transplants were treated with TGF-β alone, RA alone, both or none. The percentage of Tregs or Th17 cells in $CD4^+$T cells, the level of FoxP3 protein or serous interleukin (IL)-17A, or suppressive function of Tregs from recipient mice were assessed. The percentage of Th17 cells and level of serum IL-17A both increased significantly during acute rejection. RA favored differentiation to Tregs over Th17 cells. Unlike naive T cells, only a few transferred iTregs remained after transfer. Treatment with RA plus TGF-β prolonged graft survival, increased the percentage of Tregs, and decreased the percentage of Th17 cells in peripheral T cells. Tregs from all recipients had normal suppressive function. In conclusion, treatment with RA plus TGF-β attenuates acute rejection by promoting the differentiation of Tregs and inhibiting the differentiation of Th17 cells.

Introduction

Regulatory T cells (Tregs) can suppress the proliferation and/or function of various immune cells, such as $CD4^+$ T cells, $CD8^+$ T cells, natural killer (NK) T cells, dendritic cells (DCs), monocytes/macrophages, B cells, and NK cells [1,2]. Forkhead box P3 transcription factor (FoxP3) is the cell lineage-determining master transcription factor for Tregs, also known as $CD4^+CD25^+FoxP3^+$ regulatory T cells'. Tregs finely tune immunity and tolerance in the periphery and are generated in the thymus. Mutation in the FoxP3 gene has been found in patients with immunodysregulation, polyendocrinopathy, enteropathy, and X-linked syndrome and indicates that Tregs play a central role in the maintenance of immunologic homeostasis and tolerance in the T-lymphocyte compartment [3,4].

Numerous studies have shown the importance of Tregs in acute allorejection, which is characterized by infiltration of the graft by donor Ag-primed T cells, their activation, and finally graft tissue destruction [5,6]. Tregs can suppress the proliferation and/or function of infiltrative T cells and relieve acute allorejection. Furthermore, Treg

numbers are noted to have increased in a mouse model of transplant tolerance [7], and adoptive transfer of induced Tregs (iTregs) can relieve rejection of heart transplants in mice [8]. However, intragraft FoxP3 expression increases in cardiac allograft patients with acute rejection [9]. Cytokines IL-2 and transforming growth factor beta (TGF-β) *in vitro* can induce FoxP3 expression in naive conventional T (Tconv) cells, which then acquire some characteristics of Tregs, including suppressive properties in some contexts [10–12]. In contrast, FoxP3 expression in TGF-β-induced Tregs is unstable [13]. These cells are not suppressive in all assays, and converted cells acquire only a partial segment of the genomic signature typical of Tregs. In addition, we also found that most iTregs disappear after transfer to congenic mice, and about one-third of the remaining iTregs lose their FoxP3 protein. Thus, the role of adoptively transferred iTregs in reducing rejection is limited, and Tregs generated *in vivo* might be more effective.

A third independent subset of effector T cells, Th17, produces IL-17 [14], which is an inducer of proinflammatory cytokines (IL-6, granulocyte colony-stimulating factor (G-CSF), and several chemokines) by fibroblasts and epithelial cells [15]. IL-17 expression was first associated with many inflammatory diseases in humans, such as rheumatoid arthritis, asthma, systemic lupus erythematosus, and allograft rejection [15–17], and is now identified with the induction/maintenance of a variety of diseases, ranging from autoimmune and inflammatory diseases to cancer and infectious diseases [18]. Although some studies confirm that IL-17 expression increases in renal transplantation [19] and lung transplantation [20], its role in transplantation is unknown. To determine whether Th17 cells play a role in transplant rejection, we monitored the changes in the level of Th17 cells and serum IL-17A during acute heart rejection.

All-trans-retinoic acid (RA) is a biologically active and key metabolite of Vitamin A and is an important morphogen that affects the development and maintenance of a wide variety of tissues, as exemplified by the pleiotropic abnormalities that appear in vitamin-A-deficient embryos or adults [21]. RA can have general stimulatory effects on lymphocyte responses, possibly by inhibiting apoptotic pathways [22]. Importantly, RA has a role in regulating reciprocal differentiation of Tregs and Th17 cells, can inhibit the IL-6-driven induction of proinflammatory Th17 cells, and can promote anti-inflammatory Treg differentiation in TGF-β-dependent immune responses [23].

Until now, regulation of the reciprocal differentiation of Tregs and Th17 cells has been studied mainly *in vitro*, but regulation *in vivo* remains unclear. Herein, our aim was to determine whether Th17 cell numbers increase during acute rejection of heart transplants, and whether RA in the presence of TGF-β can relieve acute rejection and prolongs graft survival, possibly by promoting the conversion of Tregs and inhibiting differentiation of Th17 cells.

Materials and methods

Animals

Adult male BALB/c (H2d), C57Bl/6J(B6-H2b), C57Bl/6J(CD45.1 congenic), and FoxP3^{gfp} mice were purchased from the Center of Experimental Animals, Tongji Medical College of HUST (Wuhan, China) or from Jackson Laboratories (Bar Harbor, ME, USA) and used at 8–10 weeks of age as recipients and donors respectively. All mice were bred in a pathogen-free environment in the above Center of Experimental Animals in accordance with the guidelines of Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International). Effort was made to ensure that the investigation conformed to the guidelines for the handling of experimental animals formulated by the Research Committees of HUST University, Wuhan, China.

Heterotopic cardiac transplant and post-transplant therapies

Donor hearts were heterotopically transplanted into recipient mice [24]. The mice were anesthetized by a single intraperitoneal injection of ketamine/xylazine (100:10 μg/kg). B6 hearts were transplanted into BALB/c recipients as allografts, and BALB/c hearts were transplanted into the same strain as isografts. Some BALB/c mice underwent sham operations. The strength and quality of cardiac impulses were graded by palpation on daily basis, as previously described [25]. For each graft, rejection of cardiac grafts was considered to be complete upon heartbeat cessation and was confirmed visually for each graft by laparotomy. A volume of TGF-β (Sigma, St Louis, MO, USA) was loaded into a model 1002 osmotic pump (ALZET Osmotic Pumps, Cupertino, CA, USA) designed to release 5 μg/kg/day (a dose based on weight of the recipient mouse) for 2 weeks, and the pump was implanted intraperitoneally. If the donor heart survived more than 13 days, the pump was replaced. Group B allograft recipients were treated with all-trans-RA (Sigma), which was dissolved in sesame oil, and intragastrically administered daily (3 mg/kg). Other allograft recipients were treated with RA (3 mg/kg/day) and received osmotic pumps containing TGF-β (5 μg/kg/day) (group C; treatment with combination of RA and TGF-β), or received osmotic pumps containing TGF-β (5 μg/kg/day) only (group D; TGF-β only), or were treated with carrier solutions (group A). The blood, spleen, and

allograft were harvested at day 5 post transplantation or at rejection.

Histology

Allografts were harvested at the times indicated post transplantation, fixed in formalin, and embedded in paraffin. Sections were stained with H&E to assess the pathologic grade, and diagnosis of acute cellular rejection was made according to the criteria of International Society of Heart and Lung Transplantation (ISHLT) Standardized Cardiac Biopsy grading (1990).

Naive $CD4^+$ T-cell purification, stimulation and culture

Naive $CD4^+$ T cells ($CD4^+CD25^-$) were purified by FACS (FACSAria, BD Biosciences, San Jose, CA, USA) following isolation of $CD4^+$ cells from BALB/c mice using magnetic beads (Dyanal beads; Invitrogen, Carlsbad, CA, USA). Naïve $CD4^+$ T cells were activated with plate-bound anti-CD3 (4 μg/ml) and soluble anti-CD28 (2 μg/ml) in 96-well plates. Activated naïve cells cultured in RPMI 1640 (GIBCO, Carlsbad, CA, USA) containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1 × 2-mercaptoethanol were stimulated with TGF-β (2 ng/ml) or IL-6 (100ng/ml) plus TGF-β (2 ng/ml) for 3 days to induce Th17 cell differentiation, and with TGF-β (1 ng/ml) for 3 days to induce Treg conversion in the presence or absence of RA (0.1 μM). Flow cytometric analysis (FACSCalibur, BD Biosciences, San Jose, CA, USA) was used to measure the percentage of Th17 and iTreg in the total $CD4^+$ T cell population.

Induction of Treg and adoptive transfer

Purified naive $CD4^+$ T cells ($CD4^+GFP^-$) from $FoxP3^{gfp}$ mice were harvested as described previously, activated with plate-bound anti-CD3 (4 μg/ml) plus soluble anti-CD28 (2 μg/ml), and for induction of iTregs, cultured in complete RPMI 1640 with TGF-β (2 ng/ml) plus IL-2 (20 U/ml) in 24-well plates for 5 days. The $CD4^+GFP^+$ T cells (iTregs) were separated from the rest of the cultured cells by FACS. The same number of iTregs and purified naive $CD4^+$ T cells (3×10^6) were respectively adoptively transferred into congenic mice. CD45.2 was used as a marker of the adoptively transferred cells 2 weeks after the transfer.

Intracellular Staining and Flow Cytometry

Splenocytes (in single-cell suspension) from allograft recipients or treated $CD4^+$ T cells were stimulated with a combination of 5 ng/ml phorbol 12-myristate 13-acetate, 500 ng/ml ionomycin (Sigma-Aldrich, St. Louis, MO, USA), and monensin (GolgiStop, 1 μl/ml; BD Biosciences) for 5 h or left unstimulated, stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD4 mAb (BD Biosciences), washed, fixed, permeabilized, and stained with PE-conjugated anti-IL-17 mAb or PE-conjugated anti-FoxP3 mAb (BD Biosciences, San Jose, CA, USA) using the BD Cytofix/Cytoperm Plus Fixation/Permeabilization kit, as per the manufacturer's instructions (BD Biosciences). Finally cell analysis was performed on the FACSCalibur and the results were analysed with WINMDI software (BD Biosciences, San Jose, CA, USA).

In vitro suppression assay and CFSE labeling

T-depleted splenocytes ($CD3^-$ cells) isolated from donor mice (B6) were irradiated with 30 Gy. Tregs ($CD4^+CD25^+$), and naive T cells ($CD4^+CD25^-$)(5×10^4/well) from allograft recipients (BALB/c) purified by FACS were added in different ratios to round-bottom 96-well plates with irradiated donor $CD3^-$ splenocytes (1.5×10^5) in complete RPMI 1640 (final volume, 200 μl). Cells were cultured at 37°C in a humidified atmosphere of 5% CO_2 for 3 days, and pulsed with 1 μCi of [^{3}H]thymidine (Amersham Pharmacia Biotech, Piscataway, NJ, USA) for the last 16 h of culture. Cells were harvested onto glass fiber filters, and the incorporated radioactivity was determined by a Wallac Betaplate liquid scintillation counter (Beckman, Fullerton, CA, USA). Results are expressed as the mean CPM ± SEM. For carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling, purified naive T cells were labeled with CFSE for 12 min. CFSE-labeled naive T cells were then cultured with Tregs and irradiated donor $CD3^-$ splenocytes or only with irradiated donor $CD3^-$ splenocytes for 3 days. Proliferation of CFSE-labeled T cells was determined by FACS analysis using a FACSCalibur flow cytometer (BD Biosciences).

Western blot and ELISA

Splenocyte pellets were resuspended in urea buffer (9 M urea, 50 mM Tris, pH 7.5), solubilized by sonication, and the protein components were resolved by SDS-PAGE (10% gel). Separated proteins were transferred onto nitrocellulose, immunoblotted using anti-FoxP3 (eBioscience, San Diego, CA, USA) and anti-β actin (AC-74; Sigma) mAbs, incubated with goat anti-mouse IgG-peroxidase conjugate (Sigma) as a second step, and the bands were visualized using an enhanced chemiluminescence kit (ECL; Amersham). Serum was collected at the times indicated post transplantation, and IL-17 was quantified using an enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instructions (eBioscience).

Immunohistochemical staining of paraffin-embedded allograft

Immunohistochemical staining on paraffin sections was performed using a standard polymer-peroxidase technique. After deparaffinization and rehydration, endogenous peroxidase activity was blocked with hydrogene peroxide (0.3 in methanol for 30 min). Heat-induced antigen retrieval was performed using 10 mM Tris-HCl and 1 mM ethylene diamine tetraacetic acid (EDTA) pH 9.0 in a microwave oven for 15 min. Then, sections were covered with serum-free protein block (Dako, Glostrup, Denmark), followed by different primary antibodies. After incubation with monoclonal anti-FOXP3 (Abcam, Cambridge, UK) or monoclonal anti-IL-17A (Biolegned, San Diego, CA, USA), and EnVision+ anti-mouse/HRP polymer were used as second step. Finally, peroxidase activity was developed using a liquid diaminobenzidine (DAB+) chromogen system (Dako). For the enumeration of FoxP3+ lymphocytes, lymphocytes were counted in three fields (×100) by two independent observers. For each sample, the mean percentage of positive cells was taken. Results were expressed as the mean ± SD of all allografts in each group.

Statistical analysis

Kaplan–Meyer curves were used to estimate graft-survival time. Other results were analysed by ANOVA followed by

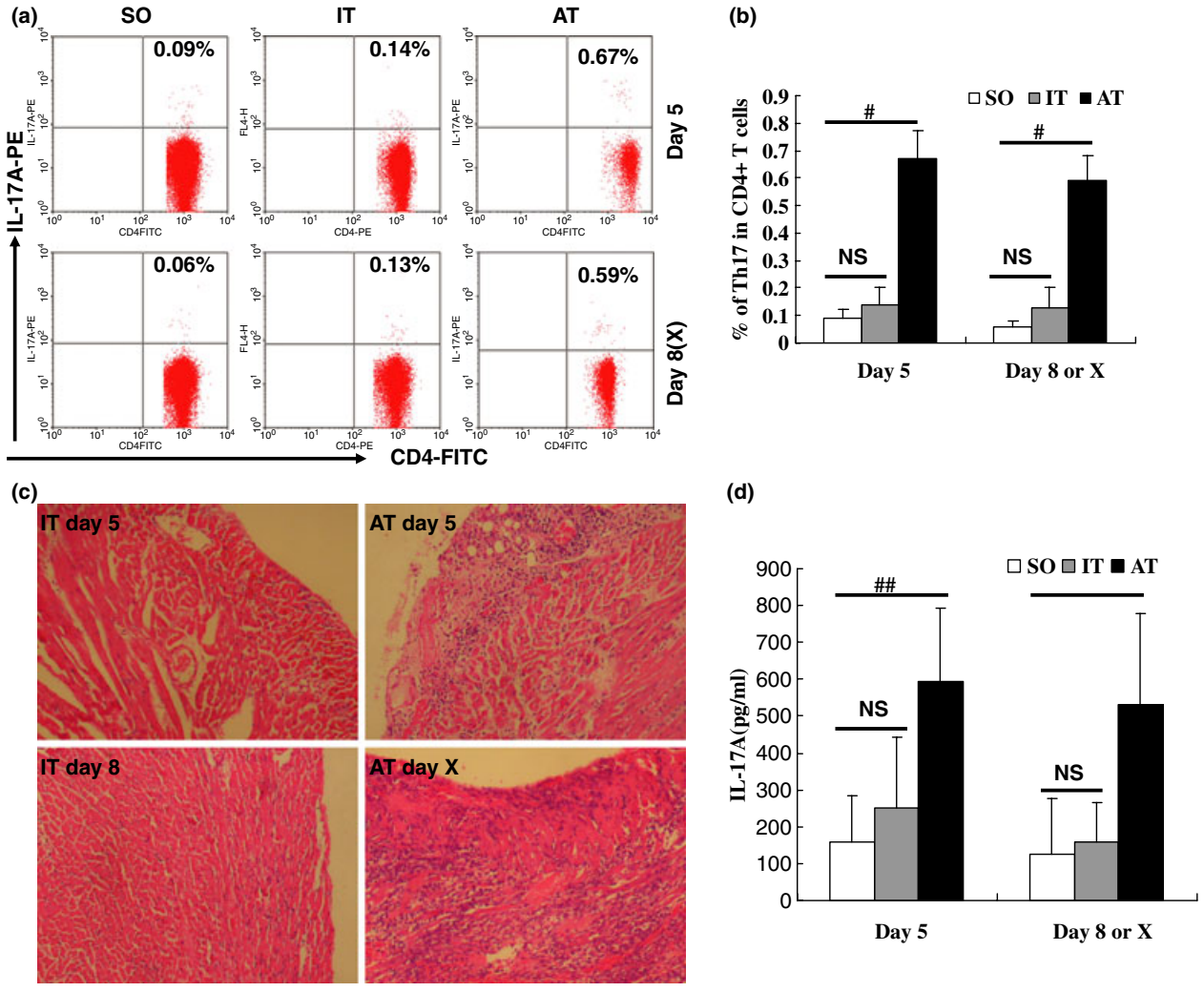


Figure 1 Percentage of Th17 cells in the CD4$^+$ T cell population, histopathology of the grafts, and level of serous IL-17A in sham operated (SO), isogeneic (IT), and allogeneic transplantation (AT) groups. (a) FACS dot plots of Th17 in CD4$^+$ gated cells on day 5 and day 8 or day X in the SO, IT, and AT groups. (b) The percentage of Th17 cells in the T cell population on day 5 and day 8 or day X in the SO (white), IT (gray) and AT (black) groups. (c) Histopathologic appearance (HE ×100) of grafts on day 5 and day 8 or day X in the IT and AT groups. (d) Level of serum IL-17A (pg/ml) on day 5 and day 8 or day X in the SO (white), IT (gray), and AT (black) groups. Day X represents the time of graft rejection. #$P \leq 0.05$.

Bonferroni correction or analysed by *t* test. A value of $P \leq 0.05$ was considered to be statistically significant. Data are expressed as mean ± SD.

Results

The percentage of Th17 cells increases during acute rejection in heart transplantation

To determine whether Th17 cells are involved in acute rejection in heart transplantation, we compared mice receiving isogeneic heart transplantation (BALB/c → BALB/c) or allogeneic heart transplantation (B6 → BALB/c) to their respective sham-operated controls. The spleen, blood, and graft were harvested on day 5 and on the day when grafts were rejected. The percentage of Th17 cells in the spleen, level of cytokine IL-17A in serum, and pathologic change in the H&E stained graft were assessed. Mice with allogeneic transplants had higher Th17 cell percentage and serum level of IL-17A than mice with isogenic transplants or sham-operated controls, these being similar in the isogeneic transplant and sham-operated groups (Fig. 1a,b and d). Likewise, pathologic change was more severe in allografts than isografts (Fig. 1c).

Differential effect of RA on the development of FoxP3$^+$ Tregs and Th17 cells *in vitro*

To assess effect of RA on the development of FoxP3$^+$ Tregs and Th17 cells *in vitro*, purified naive CD4$^+$ T cells were cultured in the presence of TGF-β alone or TGF-β plus IL-6 with or without RA. RA increased the percentage of cells expressing FoxP3 induced by TGF-β from 60.02 to 82.15 and significantly inhibited the differentiation of Th17 cells by TGF-β plus IL-6 (i.e., reduced the percentage of Th17 cells from 12.72 to 6.06). However, in the absence of IL-6, neither TGF-β nor TGF-β combined

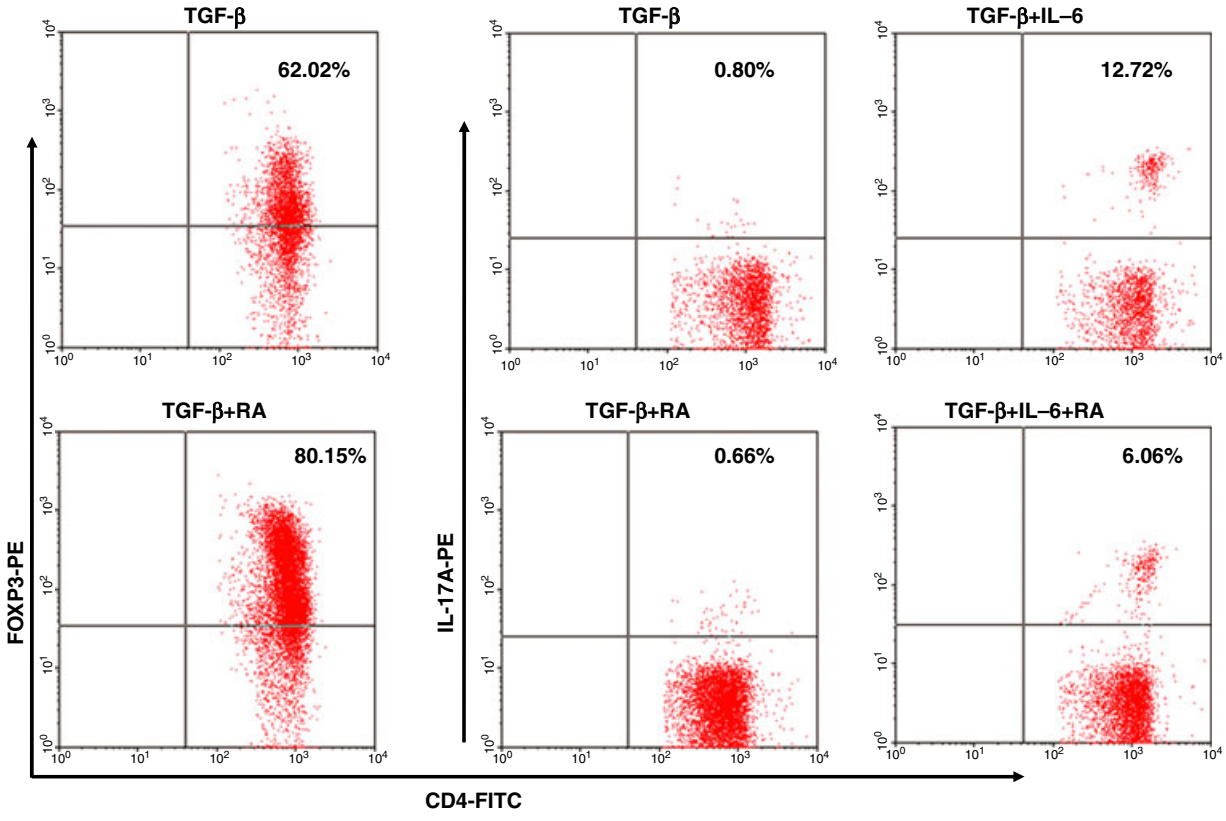


Figure 2 Differential effect of retinoic acid (RA) on the development of FoxP3$^+$ Tregs and Th17 cells *in vitro*. Naive CD4$^+$CD25$^-$ T cells purified by magnetic activated cell sorting (MACS) and FACS were activated by exposure to precoated anti-CD3 mAb (4 μg/ml) and soluble anti-CD28 mAb (2 μg/ml) for 3 days under indicated conditions. Aliquots of cells cultured with TGF-β (1 ng/ml) plus RA (0.1 μM) or DMSO were fixed, permeabilized, stained intracellularly with PE-FoxP3 mAb, and analysed by flow cytometry (left). Aliquots of cells were cultured with RA (0.1 μM) or DMSO containing TGF-β (2 ng/ml) alone or TGF-β (2 ng/ml) plus IL-6 (100 ng/ml) for 3 days, reactivated with phorbol 12-myristate 13-acetate (PMA) and ionomycin for 4 h in the presence of GolgiStop, stained with PE-IL-17 mAb, and analysed by flow cytometry (middle and right). Data are representative of two experiments.

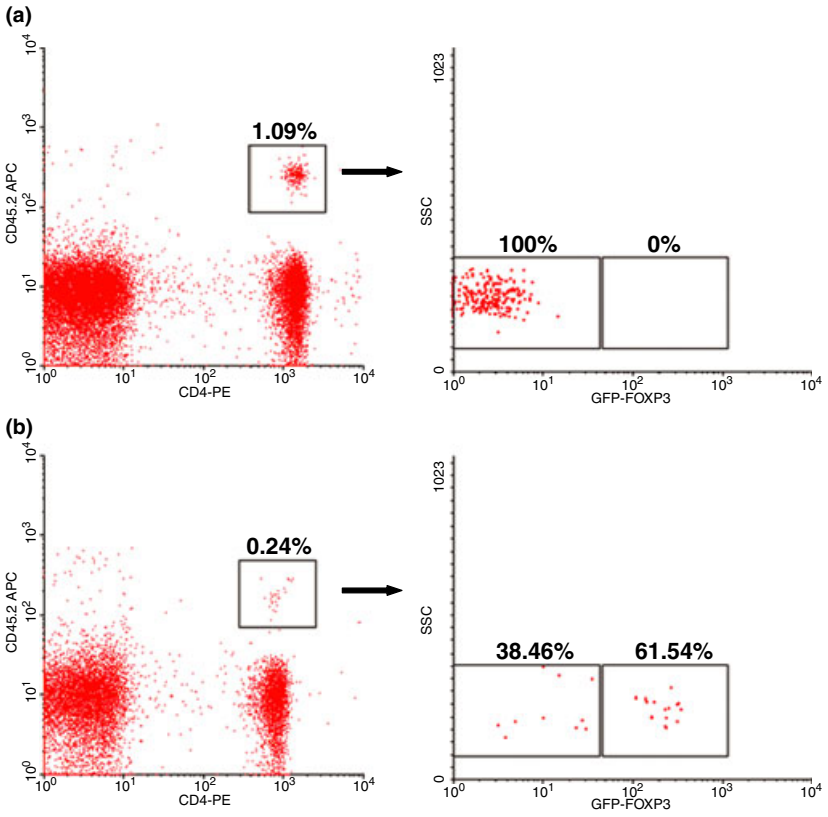


Figure 3 Loss of FoxP3 protein by adoptively transferred iTreg cells *in vivo*. Naive $CD4^+GFP^-$ T cells purified by FACS were cultured in complete RPMI 1640 with TGF-β (2 ng/ml) plus IL-2 (20 U/ml) for 5 days. The $CD4^+GFP^+$ T cells (i.e., iTregs) were purified again by FACS. Naive $CD4^+GFP^-$ T cells (3 million cells) (panel a) and $CD4^+GFP^+$ T cells (the same number of cells) (panel b) were respectively injected into C57Bl/6J congenic mice from through the penile vein, which only expressed CD45.1 marker not CD45.2 marker. Two weeks later, the adoptively transferred cells in spleen were assayed using CD45.2 as marker, and the percentage of GFP-$FoxP3^+$ cells in the $CD45.2^+$ population was calculated. Each group had three mice, and the mean percentage of $CD45.2^+CD4^+$ cells in both groups was, respectively, 1.10 ± 0.15 and 0.26 ± 0.08, $P < 0.01$. The mean percentage of GFP-$FoxP3^-$ cells in the $CD45.2^+CD4^+$ population of the iTreg group was 34.52 ± 9.16, while that of the naive T cell group was 100%.

with RA could induce the differentiation of Th17 cells (Fig. 2). Taken together, our *in vitro* results confirmed the differential role of RA in the development of Tregs and Th17 cells.

Lower survival rate and loss of FoxP3 protein of adoptively transferred Tregs *ex vivo* as compared with Tregs induced *in vivo*

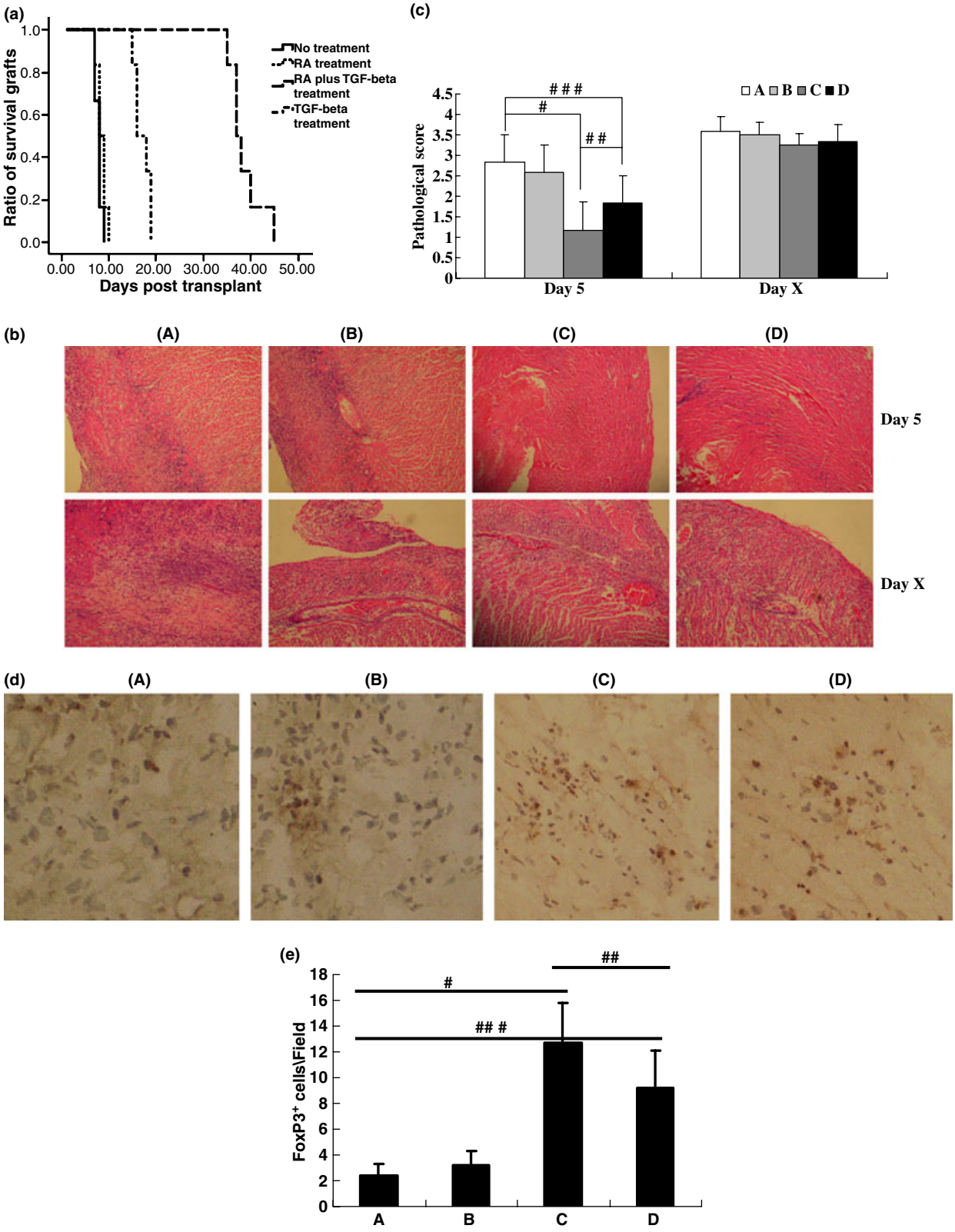
To determine the efficiency of transferred iTregs, *ex vivo* induced-Tregs or naive CD4+ T cells were adoptively transferred to different congenic B6 mice, and cell transfer was monitored 2 weeks later. The percentage of transferred iTregs in spleen was significantly lower than that of naive $CD4^+$ T cells 2 weeks later (respectively, 1.10 ± 0.15, 0.26 ± 0.08, $P < 0.01$), and 34.52 ± 9.16% of the surviving transferred iTregs had lost their FoxP3 protein, but no surviving transferred naive T cells had FoxP3 (Fig. 3).

RA alleviated acute rejection in heart transplantation and prolonged survival time of allograft in presence of TGF-β

To assess whether RA alleviated acute rejection and prolonged allograft survival, mice with allogeneic transplants were subjected to four different treatments. RA alone did not attenuate acute rejection or prolong allograft survival (Fig. 4). However, TGF-β alone markedly prolonged survival ($P<0.05$), and Treatment with combination of RA and TGF-β prolonged survival still further ($P<0.05$) (Fig. 4). By immunohistochemical staining for FoxP3 and IL-17A within paraffin-embedded allograft sections, more $FoxP3^+$ cells immigrated into the allografts in TGF-β group and the group treated with combination of RA and TGF-β compared with control group or RA alone group, and then treatment with combination of RA and TGF-β could promote more $FoxP3^+$ cells into allografts than TGF-β alone (Fig. 4D and E). However, we did not detect the $IL\text{-}17A^+$ cells in allografts (data not show).

RA relieved acute rejection by increasing the number of $CD4^+$ $FoxP3^+$ regulatory T Cells and repressing differentiation to Th17 cells *in vivo* in the presence of TGF-β

To probe the mechanism further, Tregs, Th17 cells, FoxP3 protein, and serum IL-17A were monitored in our four groups (A – no treatment; B – RA treatment; C – treatment with combination of RA and TGF-β; D – TGF-β treatment). *In vivo*, treatment with combination of RA



and TGF-β significantly increased the percentage of Tregs and expression of FoxP3 protein but decreased the percentage of Th17 cells and level of serum IL-17A (Fig. 5). The similar but weaker effect of TGF-β alone on Tregs (as compared with treatment comprising combination of RA and TGF-β) might be explained by TGF-β-mediated suppression of T-cell proliferation (a mechanism that also explains the suppressive function of Tregs [Fig. 5]), but TGF-β alone could not repress differentiation to Th17. Maintenance of a stable, higher level of TGF-β *in vivo* by the osmotic pump may account for the even stronger effect of treatment with combination of RA and TGF-β.

Treg cells from recipient mice had a normal level suppressive function

To determine whether increasing the numbers of *in vivo* Tregs contributed to the relief of acute rejection, purified Tregs from spleens in different groups were assayed by a mixed lymphocyte/leukocyte reaction (MLR) with stimulator cells representing an alloantigenic challenge. The results suggested that Tregs had normal suppressive function regardless of treatment (Fig. 6). Tregs induced *in vivo* were as functional as natural Tregs (nTregs), while Tregs induced *in vitro* were less suppressive than nTregs [9–11]. These findings suggest that *in vivo* iTregs were more suppressive than *in vitro* iTregs.

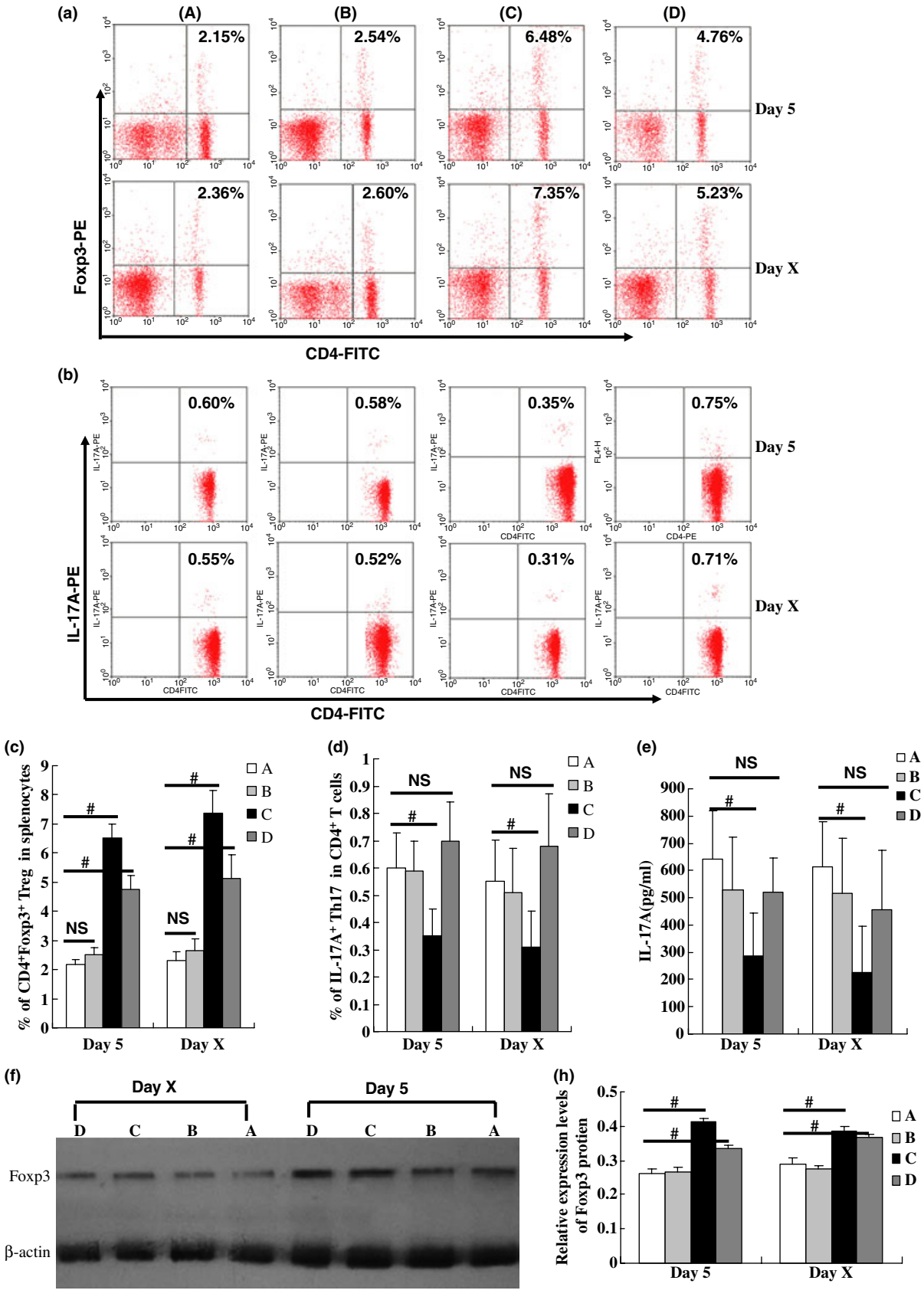
Discussion

In this study, Th17 cells are shown to be involved in acute rejection of transplanted heart. Th17 cells produce IL-17 and are principally involved in inflammation and autoimmune disease, but recently some studies found increased expression of IL-17 in lung and renal grafts [20,26] and suggested the involvement of IL-17 in vascular rejection [27]. IL-17 is a strong pro-inflammatory cytokine that stimulates different cells to secrete and release IL-6, IL-8, granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF), chemokine ligand (C-X-C motif) 1 (CXCL1), chemokine ligand (C-X-C motif) 10 (CXCL10), and monocyte chemotactic protein 1 (MCP-1) [17,28]. In the late stage of acute rejection, cells are attracted to the graft by inflammatory factors and destroy the tissues. IL-17 antagonism prolongs graft survival [29]. In this study, we confirmed that IL-17A level increases during acute rejection, and the number of Th17 cells in lymphoid tissue also increases, but no Th17 cells were present in the graft (data not shown)[30], which is probably ascribed to Th17 accounting for a small part of the T cell population.

Regulatory T cells (Tregs) are suppressor T cells and specifically express FoxP3 protein. Tregs are divided into two types: induced Tregs and naturally occurring Tregs. The latter generated in the thymus have a stable suppressive function, while the former generated in the periphery or *in vitro* have an unstable or transient suppressive function. Adoptive transfer of iTregs prolongs and even prevents graft rejection in transplantation models [8,31,32]. Therefore, we monitored changes in *in vitro*-induced Tregs transferred from FoxP3^{gfp} mice to congenic mice. Unlike transferred naive T cells, four-fifths of transferred iTregs died and disappeared, and one-third of surviving iTregs lost their FoxP3 protein 2 weeks later. In mice, only repeated transfer of large numbers of iTregs could ameliorate acute rejection. The reason may be that *in vitro* iTregs (unlike resting naive T cells) are activated, and more cytokines are needed to keep the cells activated. However, the *in vivo* environment provides insufficient amounts of cytokines, so that only a few activated cells can remain alive. This physiologic phenomenon is thus akin to the generation of memory T cells.

Prolonging the persistence of Tregs *in vivo* will maintain their proportion and suppressive function, and in turn prolong survival of the graft and even induce transplantation tolerance. The vitamin A metabolite, RA, has recently been shown to enhance TGF-β-mediated FoxP3 expression and Treg cell conversion [23,33,34] and to decrease TGF-β-mediated Th17 differentiation and IL-17 production [23]. But RA alone is not sufficient for conversion to FoxP3$^+$ Tregs *in vitro* and requires the presence of TGF-β. Some findings suggested that RA enhanced TGF-β signaling by increasing the expression and phosphorylation of Smad3, and also inhibited the expression of IL-6Ralpha, IRF-4, and IL-23R, and these resulted in increased expression of FoxP3 and decreased generation of Th17 cells [35]. Other findings suggested that RA reduced STAT6 binding to the FoxP3 promoter or indirectly relieved inhibition of

Figure 4 Effect of retinoic acid (RA) or/and TGF-β on allograft rejection. (a) Survival curves for the four different treatment groups. (b) Pathologic changes of different allografts by H&E staining (×100). (c) Pathologic scores were used to estimate pathologic changes. (d) Immunohistochemical staining for FoxP3 within paraffin-embedded allografts of four groups at day X, and positive cells were stained brown or tan color in cell nuclei. (e) The average numbers of positive cells of every group in one visual field. A–D markers in panels B–E show different groups from the no treatment group, RA treatment group, RA plus TGF-β treatment group, and TGF-β treatment group respectively. Day X indicates the time when allografts were rejected. #$P = 0.019$, ##$P = 0.000$ and ###$P = 0.045$ among different groups.



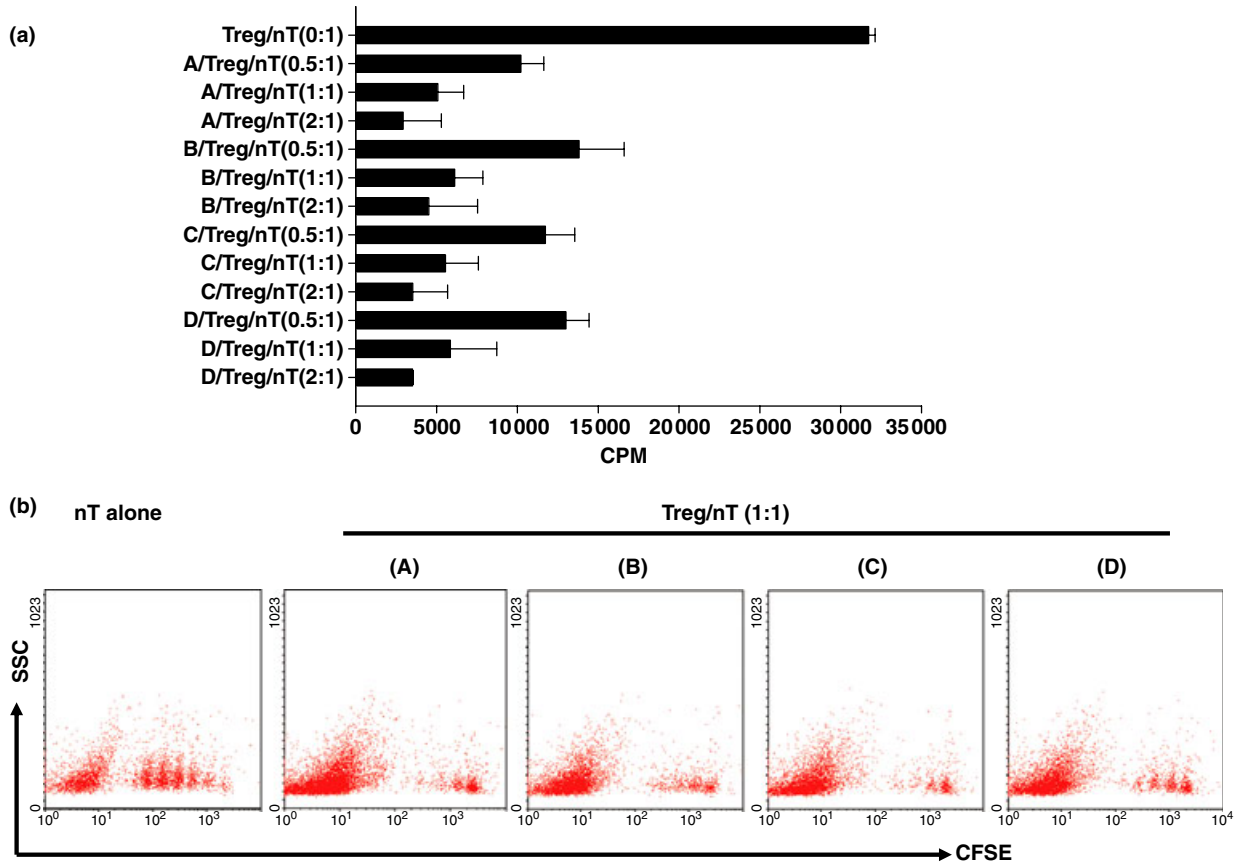


Figure 6 The effect of retinoic acid (RA) on the Treg suppressive function *ex vivo*. The spleen $CD4^+CD25^-$ T cells (naive T cells, nTs), $CD4^+CD25^+$ cells (regulatory T cells, Tregs), and $CD3^-$ splenocytes (antigen-presenting cells, APCs) were separated by FACS sorting. (a) Different ratios of Tregs to nTs to donor APCs (2:1:3, 1:1:3, and 0.5:1:3) were incubated 56 h with irradiated donor APCs (1×10^5). After incubation with [^{3}H]-thymidine (1 μCi/well) for 16 h, CPM was determined. Data are the means and SEM of at least six samples from three independent experiments. (b) Purified naive T cells were labeled with CFSE for 12 min. CFSE-labeled naive T cells were then cultured with Tregs and irradiated donor APCs (1:1:3) or only with irradiated donor APCs (1:0:3) for 3 days. Proliferation of CFSE-labeled T cells was determined by FACS analysis using a FACSCalibur flow cytometer.

TGF-β-mediated Foxp3 expression from cytokine-producing CD4+CD44hi Cells and finally enhanced FoxP3 expression [36,37]. However, the mechanism is not clear. Xiao *et al.* [35] confirmed that RA enhanced TGF-β-signaling by increasing the expression and phosphorylation of Smad3, and this resulted in increased expression of FoxP3. RA also inhibited the expression of IL-6Ralpha, IRF-4, and IL-23R and thereby the generation of Th17 cells. Furthermore Hill *et al.* [36] proposed that RA enhanced Foxp3 induction indirectly by relieving inhibition from cytokine-producing $CD4^+$CD44hi Cells, which actively restrained TGF-β-mediated Foxp3 expression in naive T cells. It was suggested that RA in the presence of TGF-β reduced STAT6 binding to the FoxP3 promoter, enhanced histone acetylation, and enhanced FoxP3 expression [37]. In EAE models, RA alone does not increase con-

Figure 5 Differential effect of retinoic acid (RA) on the development of $FoxP3^+$ Tregs and Th17 cells in mouse heart transplant recipients. (a) or (b) FACS dot plots showing the percentage of $CD4^+FoxP3^+$ Tregs in splenocytes or the percentage of $CD4^+$IL-$17A^+$ Th17 in $CD4^+$ T cells on day 5 and day X in A–D groups, and every dot plot was near or equal to the average. (c) or (d) Histogram showing the average percentages of Tregs or Th17 cells which were calculated from the dot plots in panel A or B. (e) Histogram showing serum level of IL-17A. (f) Western blot showing the expression of FoxP3 protein on day 5 and day X in A, B, C, and D groups. (h) Histogram showing the expression level of FoxP3 protein relative to expression level of endogenous β-actin protein. A – no treatment group; B – RA treatment group; C – RA plus TGF-β treatment group; D – TGF-β treatment group. Day X is the time when allografts were rejected. Data are the means and SEM of six samples from each group. #$P \leq 0.05$ between two groups. NS, $P > 0.05$ between two groups.

version to Tregs, but inhibits generation of Th17 cells [35]. *In vivo*, the level of TGF-β is too low to promote Treg conversion even in presence of RA. In this study, high exogenous TGF-β level was maintained over a prolonged period by release from an osmotic pump. But TGF-β alone induced FoxP3 expression, and directly inhibited proliferation *in vitro*. We maximized the suppressive function of Tregs by combining TGF-β and RA, and thereby ameliorated acute rejection and prolonged graft survival. The combination significantly increased the percentage of Tregs and expression of FoxP3, while markedly decreasing the percentage of Th17 cells and level of serum IL-17A. Finally, we found the suppressive function of Tregs was normal and similar in all mice irrespective of their treatment. In addition, we found RA alone did not mitigate acute rejection. However, in a previous report, RA receptor-alpha-selective agonist plus cyclosporine A relieved cardiac allograft vasculopathy in chronic rejection of heart transplantation by inhibiting proliferation of smooth muscle cells [38]. Further study of the function of treatment with combination comprising RA and TGF-β in chronic rejection is still needed.

In summary, this study confirmed that Th17 cells are probably involved in acute rejection of heart transplants. Adoptively transferred, *in vitro*-induced, Tregs readily die and lose their FoxP3 protein. However, Tregs *in vivo* induced by treatment with combination of RA and TGF-β ameliorate acute rejection and prolong graft survival more effectively. Treatment with combination of RA and TGF-β may act by regulating the conversion of Tregs and differentiation of Th17 cells during acute rejection.

Authorship

GW, AZ, ZS, JX participated in research design; GW, AZ, ND, JX participated in or directed the writing of the paper; GW, AZ, SW participated in cellular part and data analysis of this work; GW participated in animal part of this work.

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