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Original Article

Aqueous extract of *Caesalpinia sappan* decelerates allograft rejection by inducing imbalance between $CD4^+$ $CD25^+$ T cells and Th17 cells

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Abstract: Objective: Aqueous extract of *Caesalpinia sappan* (CSE) has immunosuppressive activities, but the mechanism remains unknown. This study was to investigate the effect of CSE on the balance between $CD4^+$ $CD25^+$ T cells and Th17 cells. Methods: Allografted Balb/c recipients were intraperitoneally treated with CSE for 14 continuous days, and the graft survival was observed. The spleen cells and peripheral blood of the recipient mice were harvested for phenotyping by flow cytometry, detection of gene expression by real-time PCR and cytokine detection by ELISA. Results: CSE prolonged skin allograft survival, increased the percentage and number of $CD4^+$ $CD25^+$ T cells, the expression of Foxp3 and STAT5 in spleen cells, the serum levels of IL-10 and TGF-β1, whereas reduced the percentage and number of Th17 cells and serum IL-17 level in Balb/c recipients. Conclusion: CSE expanded $CD4^+$ $CD25^+$ T cells and decreased Th17 cells *in vivo* thereby improving skin allograft survival in mice, indicating that CSE affects the balance between $CD4^+$ $CD25^+$ T cells and Th17 cells in the graft to induce rejection.

Keywords: *Caesalpinia sappan*, skin allograft transplant, T-lymphocytes, regulatory T cells, Th17 cells, cytokines

Introduction

Organ transplantation is significantly limited by graft rejection. Non-specific immunosuppressive agents have severe side effects in clinical practice and thus it is imperative to develop novel immunosuppressants for the prevention of graft rejection after transplantation.

Caesalpinia sappan has been used traditionally in the treatment of a large number of diseases due to its a wide variety of medicinal properties. Several studies have found that *C. sappan* extract (CSE) can be used to treat tumor, ascites, leukemia, and arteriosclerosis, and the prtosappanin derived from *C. sappan* can prolong the survival of heart allografts in rats and prevent immune-mediated tissue damage [1]. However, the immunosuppressive effect of aqueous CSE is unknown. CSE could negatively regulate the immune responses and prolong the allograft survival, although the mechanism remains unknown.

$CD4^+$ T cells, including Th1, Th2, Th17, and regulatory T (Treg) cells, play important roles in the immune regulation. The transcription factor Forkhead box P3 (Foxp3) is important for the growth and function of $CD4^+$ $CD25^+$ Treg cells, and it has been recognized as a specific marker of Treg cells [2, 3]. Treg cells are able to inhibit the proliferation of effector T cells and the generation of pro-inflammatory cytokines by these cells; Treg cells have anti-inflammatory activity and are able to control autoimmune diseases by releasing interleukin-10 (IL-10) and transforming growth factor-β (TGF-β) [4]. Treg cells are important in preventing autoimmune responses and maintaining immune balance. Th17 cells may regulate the expression and secretion of cytokines such as interleukin 17 (IL-17) and are related to the occurrence and development of inflammatory and autoimmune diseases. Therefore, the balance between Treg cells and Th17 cells is important for the adaptive immune system. The imbalance between

Treg cells and Th17 cells may lead to autoimmune diseases, cancers, inflammation, and transplant rejection [5]. Furthermore, many studies have revealed that Treg cells and Th17 cells participate in the rejection after kidney, heart, and skin transplantation, as well as GVHD [6-9].

Therefore, we speculate that the negative regulatory effect of CSE on allotransplantation is related to the balance of $CD4^+$ $CD25^+$ T cells and Th17 cells. In this study, flow cytometry and enzyme-linked immunosorbent assay (ELISA) were employed to detect $CD4^+$ $CD25^+$ T cells/Th17 cells and serum levels of IL-10, TGF-β1, and IL-17, aiming to explore the mechanisms underlying the regulatory effects of CSE on the post-transplantation rejection.

Materials and methods

Mice and reagents

Specific pathogen-free female C57BL/6 mice (H-2b, donor, 18-22 g) and Balb/c mice (H-2d, recipient, 18-22 g) were purchased from the Experimental Animal Center of Shandong Province. Animals were maintained under standard conditions and fed with sterilized rodent food and water according to the laboratory animal care principles and the guide for the care and use of laboratory animals in our institution. The experiments were approved by the local animal use committee. Cyclosporine (CsA) was purchased from Fluka BioChemika. Antibodies purchased from eBioscience (San Diego, CA) and consisted of FITC-anti-CD4, PE-anti-CD25, and APC-anti-IL-17.

Primers for GAPDH and Foxp3 were designed by Shanghai Sangon Biological Engineering and Technology Service Co. Ltd. The mouse IL-10, TGF-β1, and IL-17 ELISA kits were from R&D Systems (Minneapolis, MN).

CSE preparation

Heartwoods of *C. sappan* were purchased from the Chinese Medicine Market (Shandong, China) and identified by the Pharmacy Faculty of Jining Medical University of China as the logwood dried heartwood part. The wood was crushed in double distilled water for 2 h. The extract was collected and mixed with six volumes of distilled water. The resultant solution was boiled for 1 h, and the liquid was collected again, mixed with distilled water, and boiled for another 0.5 h. The extract of three extractions was mixed, and the resultant solution was stored at 4°C overnight. The aqueous extract was prepared after centrifugation at 3000 r/min for 30 min. The extract was used at 37.5 g/kg for following experiments. The components of crude extract from *C. sappan* had poor activities, but the activities of components of aqueous extract were greatly improved, accompanied by increased toxicity. In our pilot study, several doses of CSE were used, and the mortality of mice was very high when the dose was higher than 37.5 g/kg.

Skin transplantation

Skin allotransplantation was performed with completely MHC-mismatched combinations of C57BL/6 donors and Balb/c recipients according to the method described by Billingham and Medawar [10]. Mice were anesthetized with pentobarbital anesthesia (50 μg/g) by intraperitoneal injection. Full-thickness trunk skin grafts of the donors were sutured onto the recipients. Bandages were removed on day 7 to visually score daily the rejection. Rejection was defined as the complete destruction or desiccation of the transplanted skin upon inspection.

Experimental groups

Balb/c recipients were divided into three groups. Mice in CSE group (n = 10) were intraperitoneally treated with CSE (37.5 g/kg) alone; mice in CsA group (n = 10) were treated with CsA (2 mg/kg) alone as a positive control; mice in control group (n = 10) were treated with an equivalent volume of saline as a negative control. Drugs were given at 3 days before transplantation by intraperitoneal injection and administered for 14 consecutive days.

Flow cytometry

A total of 1×10^6 spleen cells were washed and re-suspended in FACS buffer containing phosphate-buffered saline (PBS), 0.5% bovine serum albumin (BSA), and 0.1% sodium azide. After blocking the Fc receptors, single-cell suspension was prepared and incubated with FITC-anti-CD4, PE-anti-CD25, and APC-anti-IL-17 monoclonal antibodies for 30 min at 4°C in

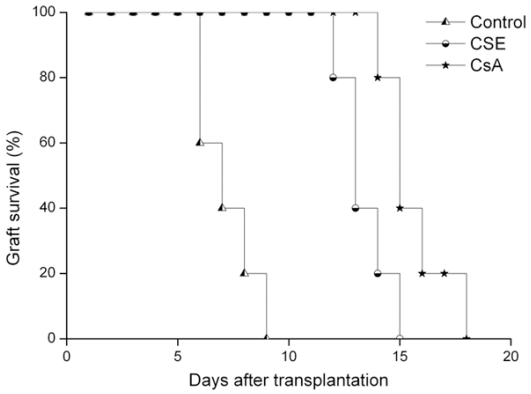

Figure 1. Skin allograft survival of mice receiving different treatments. When compared with control group, the graft survival was significantly prolonged in CSE and CsA groups (P<0.01).

dark. After washing three times, cells were resuspended in PBS containing 0.1% BSA for flow cytometry (FACSCalibur instrument; Becton Dickinson, San Diego, CA, USA). Data were analyzed with CellQuest software.

FoxP3 and STAT5 expression by real-time-PCR

Briefly, splenocytes which were harvested at 3 days and 7 days after transplantation from recipients and total RNA was extracted from 1 × 10^6 splenocytes by using Trizonal (Invitrogen) according to the manufacturer's instructions. Reverse transcription of cDNA was performed using SYBR Premix Ex Taq II (TaKaRa, Dalian, China) with random primers. PCR amplification was performed for 30 cycles at 95°C for 3 min for denaturing, 58°C (GAPDH and FoxP3) or 58.4°C (STAT5) for 30 s for annealing and then 72°C for 30 s for extension. Samples were detected in triplicate, and the relative expressions of FoxP3 and STAT5 were determined by normalizing them with GAPDH expression. For quantification, the relative mRNA expression of specific genes was obtained with $2^{-\Delta\Delta Ct}$ method. The primer sequences were as follows: GAPDH: 5'-GTGGAGATTGTTGCCATCAACG-3' (forward), 5'-CAGTGGATGCAGGGATGATGTTCTG-3' (reverse); FoxP3: 5'-CAGCTGCCTACAGTGCCCCTAG-3' (forward), 5'-CATTTGCCAGCAGTGGGTAG-3' (reverse) [4]; STAT5: 5'-ATTACACTCCTGTACTTGCGA-3' (forward), 5'-GGTCAAACTCGCCATCTTGG-3' (reverse) [11].

Cytokine detection

Peripheral blood was collected from the ear vein of recipients at 3 days and 7 days after transplantation. Blood was stored at room temperature for 30 min and then centrifuged at 3000 rpm for 10 min. The serum levels of IL-10, TGF-β1, and IL-17 were detected by sandwich ELISA according to the manufacturer's recommendations. Samples were assayed in triplicate and the serum levels of these factors were calculated according to the standard curves.

Statistical analysis

Data are expressed as mean ± standard error (SEM). The differences in $CD4^+$ $CD25^+$ T cells, expressions of FoxP3 and STAT5 as well as the serum levels of cytokines were compared using two-tailed unpaired student *t* test. Graft survival was analyzed using the Kaplan-Meier method, and compared with log-rank test between groups. A value of $P<0.05$ was considered statistically significant.

Results

Effects of CSE on the skin allograft survival

The median survival time of skin allograft was 7.2 days in control group, 13.4 days in CSE group and 15.6 days in CsA group (**Figure 1**). The survival time in CSE and CsA groups was significantly longer than that in control group ($P<0.01$). However, no significant difference was found between CSE group and CsA group ($P>0.05$).

Effects of CSE on $CD4^+$ $CD25^+$ T cells

$CD4^+$ $CD25^+$ T cells are key cells in maintaining immunological self-tolerance and immune responses. The effect of CSE on $CD4^+$ $CD25^+$ T cells was investigated in the present study. On days 3 and 7 after transplantation, splenocytes from Balb/c recipients were harvested and detected by flow cytometry. As shown in **Figure 2**, on days 3 and 7 after transplantation, the numbers of $CD4^+$ $CD25^+$ T cells in CSE and CsA groups significantly increased when compared with control group ($P<0.01$). However, these numbers in CSE group were markedly lower than those in CsA group ($P<0.01$).

Effects of CSE on Foxp3 and STAT5 expressions

To investigate the effects of CSE on the Foxp3 and STAT5 expressions, splenocytes were collected from recipient mice, and the Foxp3 and

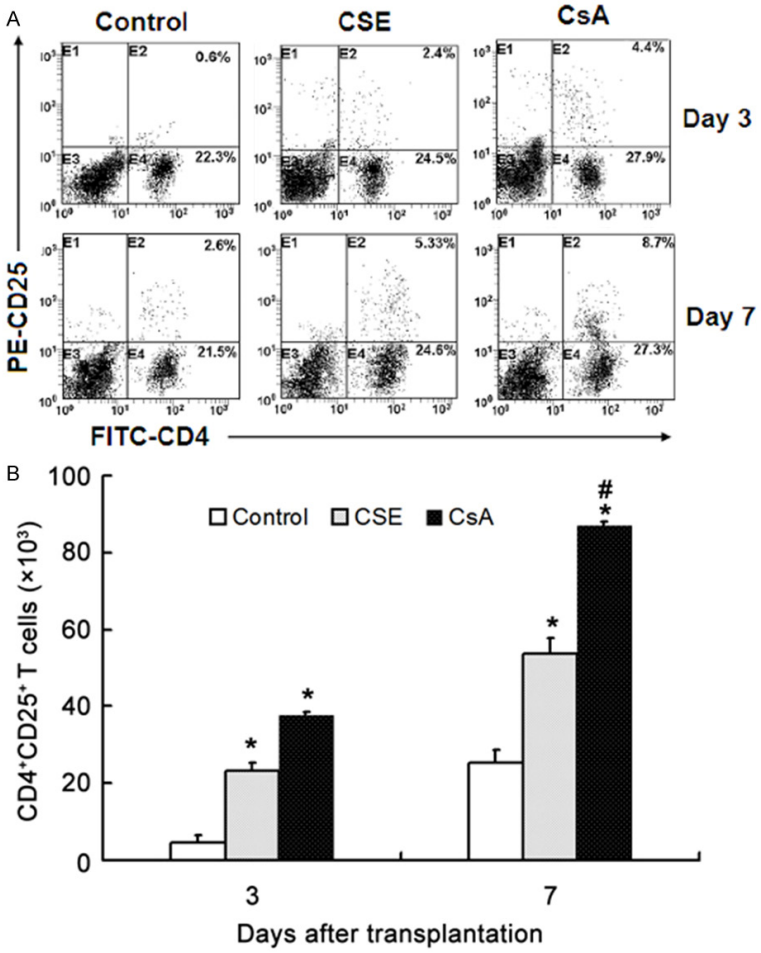

Figure 2. CSE increases the number of $CD4^+$ $CD25^+$ T cells. Splenocytes of Balb/c recipients were collected on days 3 and 7 after transplantation. The $CD4^+CD25^+$ T cells were detected by flow cytometry: A: Proportion of $CD4^+CD25^+$ T cells on day 3 and day 7 after transplantation; B: Number of $CD4^+$ $CD25^+$ T cells on days 3 and 7 after transplantation. Splenocytes were collected from mice in three groups and subjected to CD4 and CD25 staining. *P<0.01 vs. control group; #P<0.01 vs. CSE group.

STAT5 expressions were detected by real-time PCR. When compared with control group, the Foxp3 (**Figure 3A**) and STAT5 (**Figure 3B**) expressions in CSE and CsA groups significantly increased (P<0.01) on days 3 and 7 after transplantation, but the Foxp3 expression in CSE group was significantly lower than that in CsA group (P<0.01) on day 7 after transplantation.

Effects of CSE on Th17 cells

Flow cytometry demonstrated that the number of Th17 cells in CSE and CsA groups were markedly lower than that in control group (*P*<0.05, **Figure 4A** and **4B**).

Effects of CSE on the ratio of $CD4^+$ $CD25^+$ T cells/Th17 cells

The ratio of $CD4^+$ $CD25^+$ T cells/Th17 cells in CSE group was significantly higher (P< 0.01) than that in control group but markedly lower (P<0.01) than that in CsA group on days 3 and 7 after transplantation (**Figure 4C**).

Effects of CSE on serum levels of IL-10, TGF-β1, and IL-17

The effect of CSE on cytokines was not clear. To detect cytokines after CSE treatment, serum of Balb/c recipients was collected on days 3 and 7 after transplantation. The serum levels of IL-10, TGF-β1 and IL-17 were determined by ELISA and results are shown in **Figure 5**. Animals treated with CsA had significantly higher serum IL-10 and TGF-β1 levels than those in control group on day 3 after transplantation (*P*<0.05). Animals treated with CSE and CsA had significantly higher serum IL-10 and TGF-β1 levels than those in control group on day 7 after transplantation (*P*<0.05). The serum IL-17 level in CSE and CsA groups dramatically reduced when compared with control group on days 3 and 7 after transplantation (P<0.05). However, no significant differences in the serum levels of IL-10, TGF-β1, and IL-17 were found between CSE and CsA groups (P<0.05).

Discussion

The present study indicated that CSE increased $CD4^+$ $CD25^+$ T cells, expressions of Foxp3, and STAT5 in spleen cells as well as serum levels of IL-10 and TGF-β1 in peripheral blood, but decreased Th17 cells and serum IL-17 level following skin transplantation in mice. These results suggest that the regulatory effects of CSE on the allograft rejection after transplanta-

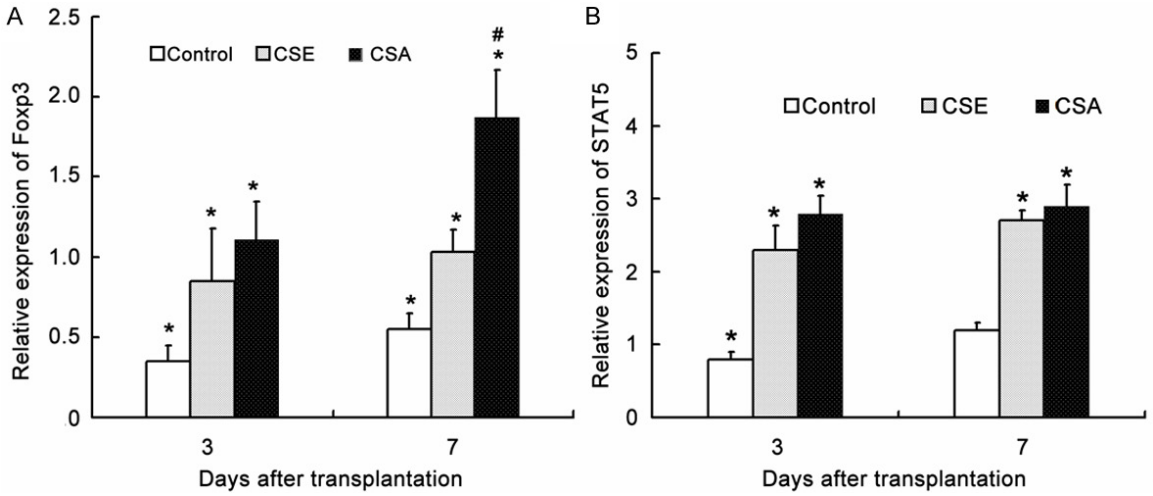

Figure 3. CSE increases the Foxp3 and STAT5 expressions. Splenocyte of Balb/c recipients were collected on days 3 and 7 after transplantation. The expressions of Foxp3 (A) and STAT5 (B) were detected by real-time PCR. Data represent the means ± SEM and are representative of two experiments. *P<0.01 vs. control group.

tion are related to the regulation of balance between $CD4^+$ $CD25^+$ T cells/Th17 cells.

Allograft acceptance/rejection is associated with multiple suppressor/effector mechanisms, and the immunosuppressants administered during transplantation have been shown to affect T cell populations. The importance of $CD4^+$ $CD25^+$ T cells and Th17 cells has become confirmed in the adaptive immune system. Treg cells and Th17 cells are derived from naïve T cells. Th17 cells mediate inflammatory reactions, whereas Treg cells mediate immune tolerance.

Given the presence of lymphocytes and dendritic populations in the skin, skin allografts that survive in the host are more resistant to treatment. Therefore, skin allotransplantation was performed to investigate the effect of CSE on the allograft survival with CsA as a positive control. CsA has been known to suppress rejection and prolong allograft survival. Our results showed that CSE prolonged allograft survival, and the efficacy was comparable between CSE and CsA.

As a traditional Chinese medicine, *C. sappan* can activate blood circulation to remove blood stasis and relieve swelling and pain. CSE significantly inhibited the functions of T-cells and B-cells in mice, and this immunosuppressive effect was more potent than that of thunder god vine. Previous studies have demonstrated that an active component of *C. sappan* L. can promote cardiac graft survival [1] and suppress the activity of infiltrating T-cells by inhibiting NF-κB activity [12]. Methanolic CSE has an immunosuppressive effect on dendritic cell-mediated expansion of alloantigen-specific Treg cells and can prolong allograft survival in rats [13]. In our study, CSE prolonged skin allograft survival. This could be explained by the increases in $CD4^+$ $CD25^+$ T cells, expressions of Foxp3 and STAT5 in splenocytes, and serum levels of IL-10 and TGF-β1 in peripheral blood, which suggest the development of $CD4^+$ $CD25^+$ T cells. Thus, CSE might inhibit the T cells and B cells, two types of cells mediating the rejection between host and MHC-mismatched graft after skin transplantation, and then increase the number of $CD4^+$ $CD25^+$ T cells. As regulatory T cells, $CD4^+$ $CD25^+$ T cells can suppress the proliferation and cytokines secretion of responsive T cells. The enhanced expressions of Foxp3 and STAT5 may prolong graft survival by maintaining the stability of $CD4^+$ $CD25^+$ T cells which then antagonize rejection by secreting certain inhibitory cytokines such as IL-10 and TGF-β1.

Several reports have demonstrated Treg cells function in clinical tolerance, in which Treg cells inhibit the activation of effector cells and control the alloresponses of the graft [14, 15]. $CD4^+$ $CD25^+$ T cells are the main cells that induce transplantation tolerance [16]. To increase $CD4^+$ $CD25^+$ T cells is able to prevent or

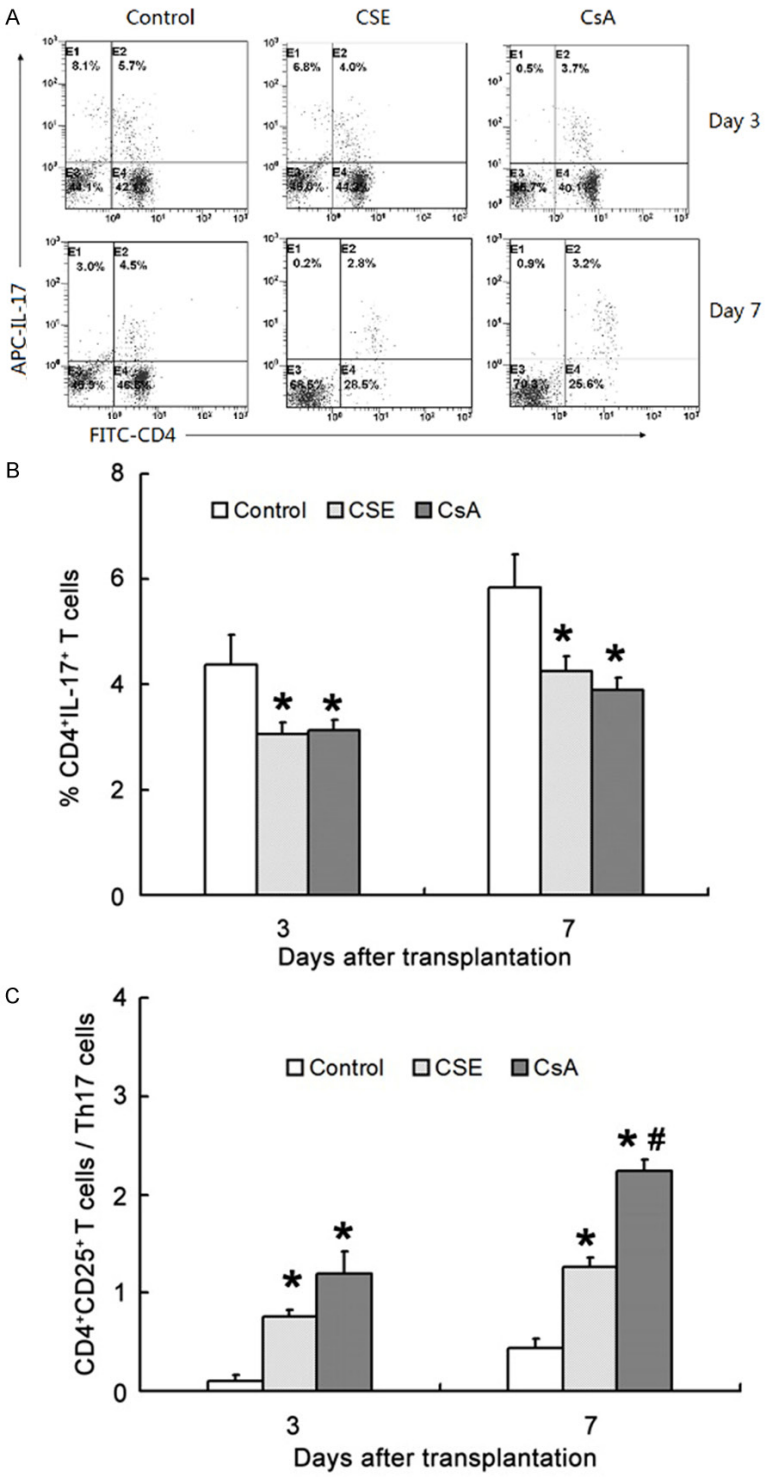

Figure 4. CSE reduces the number of Th17 cells and increases the ratio of $CD4^+$ $CD25^+$ T cell/Th17 cell. Splenocyte of Balb/c recipients were collected on days 3 and 7 after transplantation. The Th17 cells were detected by flow cytometry: A: Proportion of Th17 cells on day 3 and day 7 after transplantation; B: Number of Th17 cells on days 3 and 7 after transplantation; C: Ratio of CD4+CD25+ T cell/Th17 cell on days 3 and 7 after transplantation. Splenocytes were collected from mice in three groups and subjected to CD4 and IL-17 staining. *P<0.01 vs. control group; #P<0.01 vs. CSE group.

cure chronic GVHD following allograft transplantation [17]. As a major transcription factor, Foxp3 has been shown to be critical for the function and development of $CD4^+$ $CD25^+$ T cell [18]. The quantitative detection of Foxp3 in the liver after biopsy in recipients after liver transplantation prove the increase in $CD4^+$ $CD25^+$ T cells in acute rejection [19]. The depletion of Foxp3 leads to the development of a lethal autoimmune syndrome and the loss of Treg-suppressive activities in mice. In Foxp3-deficient mice, the adoptive transfer of $CD4^+$ $CD25^+$ T cells from wild-type mice prevents the autoimmune diseases [18, 20, 21]. STAT5 expression is also required to maintain homeostasis and Treg self-tolerance [22]. In STAT5-deficient mice, the number of Tregcellsdecreases,andinIL-2-deficient mice, the transient activation of STAT5 increases the Treg cells in the peripheral blood. Otherwise, $CD4^+$ $CD25^+$ T cells inhibit the proliferation of T and B lymphocytes in an antigen-nonspecific way [23]. $CD4^+$ $CD25^+$ T cells may secrete a variety of inhibitory cytokines, such as IL-10 and TGF-β [24], which may inhibit the functions of antigen-presenting cells and T-cells, but play important roles in the cytogenesis, proliferation, and immune tolerance induction of $CD4^+$ $CD25^+$ T cells [25, 26].

However, in our study, results showed decreases in spleen Th17 cells and the serum IL-17 level in peripheral blood, confirming the presence of Th17 cells. In agreement with our results, Hester et al. found that, in the follow-up of kidney

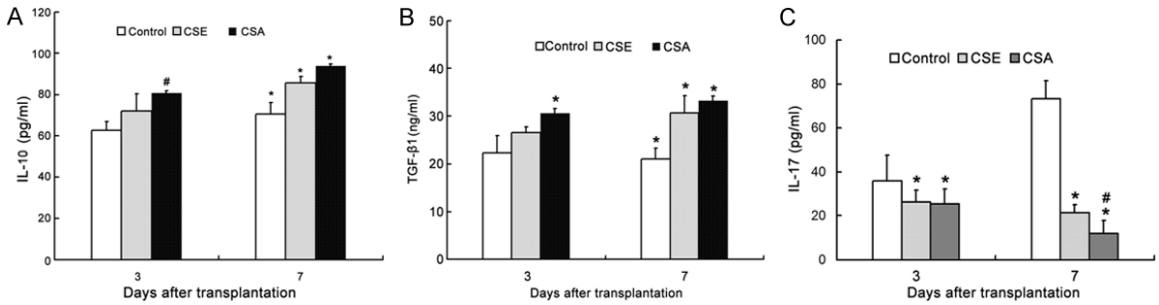

Figure 5. Effects of CSE on the serum levels of IL-10, TGF-β1 and IL-17. ELISA was performed to detect serum IL-10 (A), TGF-β1 (B), and IL-17 (C) in Balb/c recipients on days 3 and 7 after transplantation. Data represent mean ± SEM from three separate experiments. *P<0.05 vs. control group.

recipients who were induced with alemtuzumab and treated post-transplantation with several drugs such as sirolimus or sirolimus + mycophenolate mofetil, the rejection and long-term maintenance of immunosuppression influence the number of circulating Treg and Th17 cells [27].

IL-17-producing $CD4^+$ T cells (Th17) are a subtype of $CD4^+$ T cells [28] and constitute a part of the normal host response to infection. Th17 cells are also associated with allograft rejection and autoimmune diseases [29]. Given their pro-inflammatory effect, IL-17 is a pleiotropic cytokine with multiple pro-inflammatory activities that are likely to be involved in either the causation or progression of inflammatory diseases and transplant rejection in humans. Th17 cells have been implicated in allograft rejection [30]; thus, the decreases in spleen Th17 cells and serum IL-17 level in peripheral blood in the present study suggested that the prolonged skin allograft survival after CSE treatment was at least in part ascribed to Th17 cells.

The Treg/Th17 cell ratio is a useful indicator for the evaluation of severity of tissue injury and renal allograft dysfunction and has been used for predicting the clinical outcome of acute T cell-mediated rejection [6]. Calcineurin inhibitors may influence the Treg/Th17 cell imbalance in patients with renal dysfunction after transplantation [31]. Our results showed that, in recipient mice treated with CSE, the frequency of $CD4^+$ $CD25^+$ T cells significantly increased, but that of Th17 cells decreased, resulting in the increase in $CD4^+$ $CD25^+$ T cells/Th17 cell ratio on days 3 and 7 after transplantation. In control group, the frequencies of $CD4^+$ $CD25^+$ T cells and Th17 cells and the serum levels of cytokines remained unchanged. These results demonstrated that CSE could change the balance between $CD4^+$ $CD25^+$ T cells and Th17 cells, which may be one of mechanisms underlying the CSE-induced attenuation of allograft rejection.

In conclusion, our study indicate that CSE increases $CD4^+$ $CD25^+$ T cells and decreases Th17 cells *in vivo*, thereby improving allograft survival in a skin transplantation model. Prolonged transplant survival is associated with high serum levels of IL-10 and TGF-β1 and low serum IL-17 level. However, the immunoregulatory effects of CSE on $CD4^+$ $CD25^+$ T cells and Th17 cells are not as strong as that of CsA, which may be explained as the impurity of CSE as a compound.

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Disclosure of conflict of interest

None.

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