Triptolide inhibits CD4+ memory T cell-mediated acute rejection and prolongs cardiac allograft survival in mice

SHUIWEI QIU and DINGLIANG LV

Department of Cardiothoracic Surgery, People's Hospital of Quzhou, Quzhou, Zhejiang 324000, P.R. China

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Abstract. There have been numerous investigations into the immunosuppressive effects of triptolide; however, its inhibitory effects on memory T cells remain to be elucidated. Using a cluster of differentiation (CD)4+ memory T-cell transfer model, the aim of the present study was to determine the inhibitory effects of triptolide on CD4+ memory T cell-mediated acute rejection and to determine the potential underlying mechanisms. At 4 weeks after skin transplantation, mouse cervical heart transplantation was performed following the transfer of CD4+ memory T cells. Mice were divided into two groups: A Control [normal saline, 30 ml/kg/day; intraperitoneal injection (ip)] and a triptolide group (triptolide, 3 mg/kg/day; ip). Graft survival, pathological examination and the corresponding International Society for Heart & Lung Transplantation (ISHLT) scores were assessed 5 days following heart transplantation, and levels of interleukin (IL)-2, interferon-γ (IFN-γ), IL-10 and transforming growth factor β1 (TGF-β1) in cardiac grafts and peripheral blood were assessed using reverse transcription-quantitative polymerase chain reaction and ELISA. The duration of cardiac graft survival in the triptolide group was significantly increased compared with the control group (14.3±0.4 vs. 5.3±0.2 days; P<0.001). Further pathological examinations revealed that the infiltration of inflammatory cells and myocardial damage in the cardiac grafts was notably reduced by triptolide, and the corresponding ISHLT scores in the triptolide group were significantly lower than those of the control group (grade 2.08±0.15 vs. 3.67±0.17; P<0.001). In addition, triptolide was able to significantly reduce IL-2 and IFN-γ secretion (P<0.01), significantly increase TGF-β1 secretion in the cardiac grafts and peripheral blood (P<0.01) and increase IL-10 secretion in the cardiac grafts. Therefore, the present study suggests that triptolide inhibits CD4+ memory T cell-mediated acute rejection and prolongs cardiac allograft survival in mice. This effect may be mediated by the inhibition of cytokine secretion by type 1 T helper cells and promotion of regulatory T cell proliferation.

Correspondence to: Dr DingLiang Lv, Department of Cardiothoracic Surgery, People's Hospital of Quzhou, 2 Zhongloudi Street, Quzhou, Zhejiang 324000, P.R. China
E-mail: lvdingliang@126.com

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Introduction

Since the first human organ transplantation was completed in 1954, transplantation has become the most effective therapy for patients with end-stage solid organ failure (1). However, even with immunosuppressive treatments, the number of graft failures following transplantation has increased, with more patients requiring secondary transplantation (2). It has previously been reported that the incidence of graft failure following secondary transplantation is significantly higher than that of primary transplantation and the duration of graft survival is significantly shortened (3). Furthermore, it has been indicated that memory T cells serve a key role in the immune responses of secondary transplantation (4). Memory T cells have a much lower activation threshold than naïve T cells, which may stimulate a more severe immune response (5,6). Thus, a number of studies have investigated the development of novel immunosuppressants for memory T cells, including 1α-25-dihydroxyvitamin D3 and anti-OX40 L monoclonal antibodies, which are effective memory T cell blockers (7,8). The results of these previous studies suggest that inhibiting memory T cells may be a viable strategy for alleviating the immune response following secondary transplantation.

Triptolide is the active component of *Tripterygium wifordii* Hook F and has been used to treat for inflammatory and immunological disorders for >30 years in China (9). Triptolide serves an immunosuppressive role in various animal models of transplantation (10), including in heart, lung, liver, renal and islet transplantations (11-15). However, to the best of our knowledge, no studies investigating the immunosuppressive effects of triptolide on cluster of differentiation (CD)4+ memory T cell-mediated acute rejection have previously been performed. The aim of the present study was to establish a CD4+ memory T-cell transfer model to recapitulate the clinical condition and verify that triptolide is an effective treatment. In addition, the present study aimed to determine the potential mechanisms of triptolide inhibition on CD4+ memory T cell-mediated acute rejection.

Materials and methods

Animals. A total of 26 BALB/c mice and 30 C57BL/6 mice were used in the present study. Mice (both BALB/c and C57BL/6 mice used in the current study were female, 6-8 weeks old and weighed 20±2 g) were purchased from the Zhejiang Chinese Medical University Laboratory Animal Research Center (Zhejiang, China). All mice were reared under sterile conditions, as follows: Rearing temperature was 18-25˚C, relative humidity of 50-70%, a 12-h light/dark cycle and *ad libitum* access to sterile food and water, fed according to Guide for the Care and Use of Laboratory Animals (16). The present study was approved by the Experimental Animal Ethics Committee of Quzhou People's Hospital (Quzhou, China).

Skin transplantation. A total of 2 BALB/C mice and 6 C57BL/6 mice were anesthetized with 10% chloral hydrate [300 mg/kg, intraperitoneally (ip)]. The full-thickness of skin (2-3 mm thick) was harvested from the lateral thorax of BALB/C donors and pruned into 3 circular pieces (diameter >1.5 cm). Following harvest of the skin, 2 BALB/C donor mice were sacrificed using cervical dislocation, and the prepared 6 skin flaps were transplanted onto the lumbar region of 6 C57BL/6 recipients, respectively (interrupted sutures; 6-8 stitches).

$CD4^+$ memory T-cell isolation and adoptive transfer. A total of 4 weeks following skin transplantation, 6 C57BL/6 recipients were anaesthetized (10% chloral hydrate, 300 mg/kg, ip) and sacrificed by decapitation. The whole spleen was harvested and $CD4^+$ memory T cells were isolated using a MagCellect Mouse Memory $CD4^+$ T cell Isolation kit (R&D Systems, Inc., Minneapolis, MN, USA), and the purity of the extracted $CD4^+$ memory T cells was found to be >95%, as previously described (17). The concentration of $CD4^+$ memory T cells was assessed by direct cell counting using cell-count boards (QiuJing Biochemical Reagents and Instruments Co., Ltd., Shanghai, China) and was adjusted to a concentration of $1x10^6$ cells/ml. The concentration-adjusted $CD4^+$ memory T cells were transferred to 24 syngeneic recipients (C57BL/6 mice) via tail vein injection (1 ml per mouse).

Heart transplantation and experimental groups. Following the transfer of $CD4^+$ memory T cells, heterotopic cardiac transplantation was performed in the mouse necks using a non-suture cuff technique. The aorta and pulmonary artery of the BALB/C donor heart were connected to the carotid artery and external jugular vein of C57BL/6 recipient mice, respectively. The 24 cardiac recipients were randomly divided into control and triptolide groups (n=12). A total of 6 cardiac recipients in each group were randomly selected and marked as survival (C) in the control and survival (T) in the triptolide groups, respectively and used to measure allograft survival. The remaining 6 cardiac recipients in each group underwent histological examination, reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and ELISA. Triptolide ($C_{20}H_{24}O_6$, molecular weight 360, purity ≥98%) was purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany) and stored at -20˚C following reconstitution with 10 mM dimethyl sulfoxide. The dose of triptolide (3 mg/kg/day) was selected on the basis of a previous study (18) and equal amounts of normal saline (Anhui ShuangHe Pharmaceutical Co., Ltd., Anhui, China) were injected into control mice. These agents were injected intraperitoneally at days 0-5, 7, 9, 11, 13 and 15 following heart transplantation.

Allograft survival and histological examination. Allograft survival was assessed by measuring heart palpation at 5:00 p.m. daily following heart transplantation and cardiac arrest was considered to be the end event of observation. Based on the results of allograft survival, the remaining 6 cardiac recipients in the control and triptolide group were sacrificed by cervical dislocation on day 5 following the heart transplantation and cardiac grafts were removed by cutting with a scalpel along the septal plane. The specimens close to the cardiac base were fixed in 10% formalin for 12 h at 4˚C prior to embedding in paraffin (Shanghai Huayi Bio-tech Co., Ltd., Shanghai, China). Paraffin-embedded specimens were cut into sections 4 μm thick and subjected to histological study by staining with hematoxylin and eosin (H&E). Sections were viewed using an optical microscope (BM2000; Shanghai Ronbio Scientific Co., Ltd., Shanghai, China) and heart rejection score was graded by examining the extent of leukocytic infiltration and the anatomical destruction of myocytes according to the International Society of Heart and Lung Transplantation (ISHLT) standard (19,20).

RT-qPCR. Graft samples close to the apex of the heart were used for total RNA isolation using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). RT-qPCR was performed using the RT-qPCR Easy™ I (One Step)-SYBR-Green I kit (cat. no. RT-02112; Chengdu Foregene Biological Technology Co., Ltd., Sichuan, China) and performed according to the manufacturer's instructions. β-actin was used as an internal control. The primer sequences used for RT-qPCR were designed using Primer Premier 5.0 software (Premier Biosoft International, Palo Alto, CA, USA) and are presented in Table I. The qPCR cycling conditions were as follows: 1 min enzyme activation at 95˚C followed by a total of 40 cycles (15 sec denaturation at 95˚C and 1 min annealing at 60˚C) and elongation at 72˚C for 1 min. Following the reaction, the Cq values were determined by measuring the fluorescence intensity in each tube and the $2^{-\Delta\Delta Cq}$ method (21) was used to calculate relative gene expression, following three replicates.

ELISA. All recipients were anesthetized with 10% chloral hydrate (300 mg/kg, ip) and peripheral blood was harvested from the posterior ocular artery prior to the removal of the cardiac graft. The blood samples were centrifuged at 3,000 x g for 20 min at 37˚C, and the collected serum was used to detect the expression of interleukin (IL)-2, interferon (IFN)-γ, IL-10 and transforming growth factor (TGF)-β1 using the corresponding ELISA kits [mouse IL-2 ELISA kit (cat. no. EM002-96), mouse IFN-γ ELISA kit (cat. no. EM007-96), mouse IL-10 ELISA kit (cat. no. EM005-96), mouse TGF-β1 ELISA kit (cat. no. EM010-96); ExCell Biology, Inc., Taicang, China]. The detection procedure was performed according to the manufacturer's protocol and a standard curve was established to measure cytokine concentration.

Statistical analysis. Data are presented as the mean ± standard deviation. The allograft survival curve was drawn

Table I. Primer sequences used for reverse transcription-quantitative polymerase chain reaction.

Gene	Primer sequences (5'-3')
β-actin	Forward: CATCCGTAAAGACCTCTATGCCAAC
	Reverse: ATGGAGCCACCGATCCACA
IL-2	Forward: GGAGCAGCTGTTGATGGACCTAC
	Reverse: AATCCAGAACATGCCGCAGAG
IFN-γ	Forward: CGGCACAGTCATTGAAAGCCTA
	Reverse: GTTGCTGATGGCCTGATTGTC
IL-10	Forward: GACCAGCTGGACAACATACTGCTAA
	Reverse: GATAAGGCTTGGCAACCCAAGTAA
TGF-β1	Forward: TGACGTCACTGGAGTTGTACGG
	Reverse: GGTTCATGTCATGGATGGTGC

IL, interleukin; IFN, interferon; TGF, transforming growth factor.

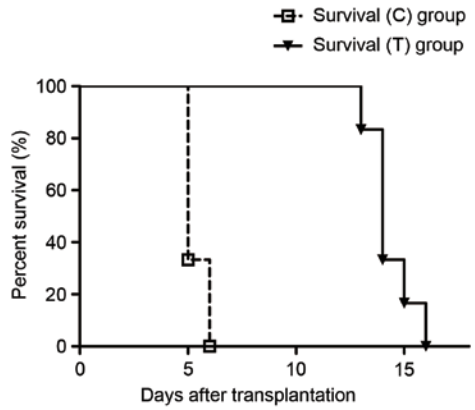

Figure 1. Triptolide prolongs cardiac allograft survival. Depending on their different immunosuppressive treatments, 6 cardiac recipients in the control and triptolide group were randomly selected and marked as survival (C) and survival (T) groups, respectively. The median survival time differed significantly between the survival (C) and survival (T) groups, at 5.3±0.2 and 14.3±0.4 days, respectively (P<0.001). Survival (C), cardiac recipients treated with saline; survival (T), cardiac recipients treated with triptolide.

using the Kaplan-Meier method and group comparisons were performed by the log-rank test. Group comparisons for RT-qPCR and ELISA detection were performed using the Student's unpaired t-test. Statistical analyses were performed using SPSS software, version 17.0 (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism, version 5 (GraphPad Software, Inc., La Jolla, CA, USA) software. P<0.05 was considered to indicate a statistically significant difference.

Results

Triptolide prolongs cardiac allograft survival. At day 5 following heart transplantation, 4 recipients in the survival (C) group experienced cardiac arrest and the 2 remaining recipients exhibited similar symptoms on day 6. Compared with the survival (C) group, recipients in the survival (T) group did not experience cardiac arrest until day 13, with 1 recipient surviving for 16 days. As presented in Fig. 1, the median survival time of the survival (T) group was significantly longer

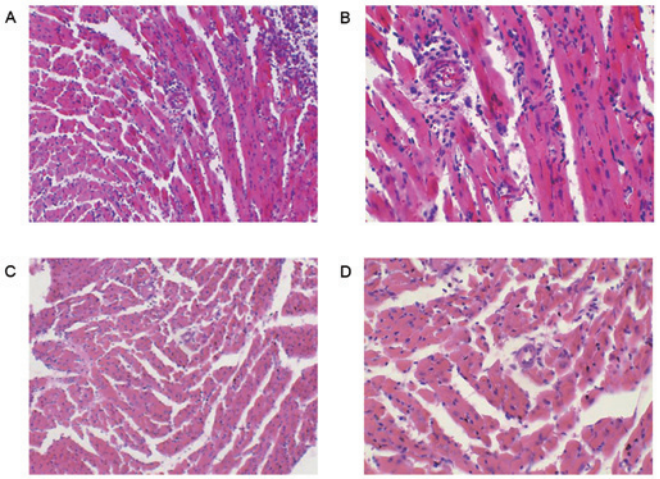

Figure 2. Pathological changes in the (A and B) control and (C and D) triptolide groups. Cardiac grafts were removed 5 days following heart transplantation and the pathological sections were observed at magnifications of (A and C) x100 and (B and D) x400. In the control group, diffuse inflammatory cells were observed in the cardiac allograft and the myocardial cells exhibited irregular arrangement, hemorrhage and necrosis. Compared with the control group, inflammatory cell infiltration in the triptolide group was reduced, and the myocardial injury was characterized by hyperemia among myocardial fibers and the partial degeneration and edema of myocardial cells.

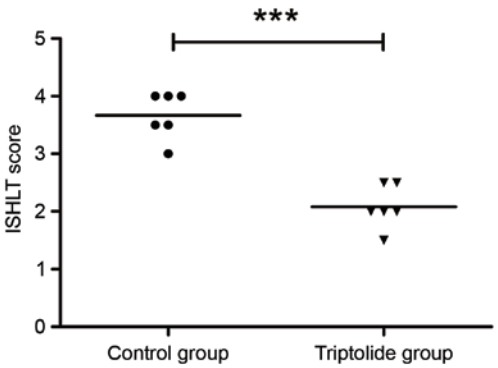

Figure 3. ISHLT scores of the cardiac allografts. Following pathological observations, the ISHLT score was calculated to assess the severity of acute rejection (6 ISHLT scores per group). The mean ISHLT score in the control group was grade 3.67±0.17, whereas the triptolide group was grade 2.08±0.15. ***P<0.001 vs. control group. ISHLT, International Society for Heart and Lung Transplantation.

than that of the survival (C) group (14.3±0.4 vs. 5.3±0.2 days; P<0.001; Fig. 1).

Cardiac allograft pathological examination and corresponding ISHLT score. The pathology of cardiac allografts was observed 5 days following heart transplantation under a light microscope at magnifications of x100 and x400. Cardiac allografts in the control group exhibited typical severe acute rejection, with pathological alterations including diffuse inflammatory cell infiltration, disordered myocardial arrangement and high levels of myocardial cell hemorrhage and necrosis (Fig. 2A and B). The corresponding ISHLT score of this section was grade 4. Compared with the control group, the severity of the acute rejection in the triptolide group was markedly reduced, a reduction in inflammatory cells and myocardial cells primarily presenting with hyperemia

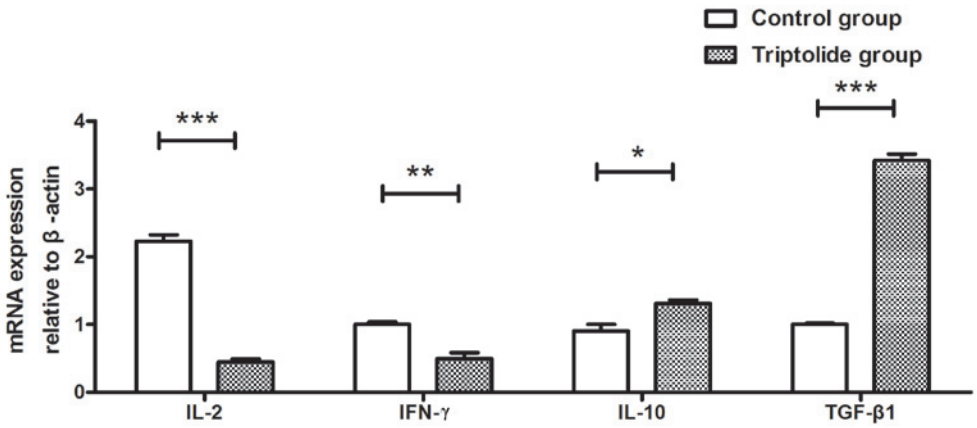

Figure 4. Levels of IL-2, IFN-γ, IL-10 and TGF-β1 mRNA in the cardiac grafts. IL-2, IFN-γ, IL-10 and TGF-β1 mRNA expression was detected 5 days following heart transplantation. In the triptolide group, levels of IL-2 and IFN-γ mRNA were significantly lower and levels of TGF-β1 mRNA was significantly higher compared with the control group. IL-10 mRNA expression also increased, although the difference between the two groups was not significant. *P<0.05, **P<0.01, ***P<0.001 vs. control group. IL, interleukin; IFN, interferon; TGF, transforming growth factor; qPCR, quantitative polymerase chain reaction.

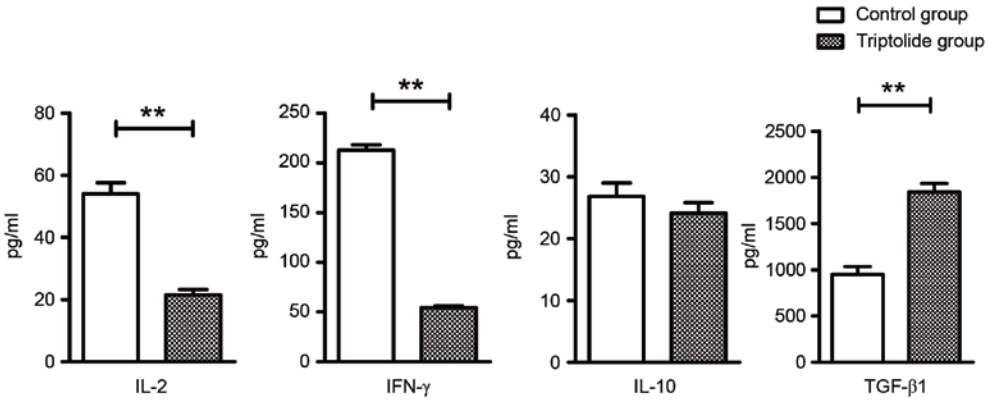

Figure 5. Levels of IL-2, IFN-γ, IL-10 and TGF-β1 in the peripheral blood. Levels of IL-2, IFN-γ, IL-10 and TGF-β1 were measured 5 days following heart transplantation. Compared with the control group, concentrations of IL-2 and IFN-γ in the triptolide group were significantly decreased and the concentration of TGF-β1 was significantly increased; however, there was no significant difference in the levels of IL-10. **P<0.01 vs. control group. IL, interleukin; IFN, interferon; TGF, transforming growth factor.

and edema, and a corresponding ISHLT score of grade 2A (Fig. 2C and D). As presented in Fig. 3, the ISHLT scores of the control and triptolide groups were grade 3.67±0.17 and 2.08±0.15, respectively. The severity of acute rejection in the triptolide group was significantly reduced compared with the control group (P<0.001; Fig. 3).

Alterations in cytokines in cardiac allograft and peripheral blood. To investigate the immunosuppressive mechanisms of triptolide, levels of the rejection-associated cytokines IL-2, IFN-γ, IL-10 and TGF-β1 were measured in the cardiac grafts and peripheral blood. Levels of IL-2 and IFN-γ mRNA in the triptolide group were significantly lower compared with the control group (IL-2, P<0.001; IFN-γ, P<0.01; Fig. 4). Levels of IL-10 and TGF-β1 mRNA also increased significantly following triptolide treatment compared with the control group (IL-10, P<0.05; TGF-β1, P<0.001; Fig. 4). Similar trends were observed in the ELISA results; concentrations of IL-2 and IFN-γ in the triptolide group were significantly reduced compared with the control group (both P<0.01; Fig. 5). A significant increase was observed in TGF-β1 levels compared with the control group (P<0.01; Fig. 5); however, levels of IL-10 did not differ significantly between the control and triptolide groups.

Discussion

Over the past few decades, it has been demonstrated that a number of Chinese herbal medicines and their derivatives, including FTY720, triptolide, genistein and arsenic trioxide (AS_2O_3) inhibit immune rejection (22-25). Recently, Ma *et al* (26) demonstrated that FTY720 was able to inhibit CD4+ memory T cell-mediated acute rejection to an extent and it was determined that the combination of FTY720 and chemokine receptor antagonists was the most efficient. Using nude mice, Li *et al* (27) reported an inhibitory effect of AS_2O_3 on CD8+ memory T cell-mediated acute rejection. Together, these studies suggest that Chinese medicine may be applied in the field of memory T-cell research. However, whilst the immunosuppressive activity of triptolide has become a topic of interest, few studies have investigated the effects of triptolide on memory T cell-mediated acute rejection. In the

present study, cardiac graft survival in the triptolide group was increased ~3-fold compared with the control group. Consistent with the survival data, histological evaluations indicated that triptolide was able to protect against inflammatory cell infiltration, reduce myocardial damage and ultimately attenuate the $CD4^+$ memory T cell-mediated immune response. To the best of our knowledge, the present study is the first to demonstrate the immunosuppressive effects of triptolide on $CD4^+$ memory T cell-mediated acute rejection and may provide evidence for expanding the application of triptolide.

Despite the well-established immunosuppressive and anti-inflammatory effects of triptolide, its inhibitory mechanisms remain to be elucidated. The mainstream view is that triptolide exerts its immunosuppressive effect by influencing the maturation and differentiation of human dendritic cells (DCs) and that a high concentration of triptolide may induce DC apoptosis (28). By contrast, a separate study indicated that the immunosuppressive effects of triptolide may be associated with the regulation of Th1-type cytokines (29). A comparative study by Qiu *et al* (30) indicated that triptolide, cyclosporine A and tacrolimus exert similar inhibitory effects on T-cell activation and IL-2 gene expression; however, the mechanism of triptolide inhibition on the IL-2 transcription site differs from that of cyclosporine A and tacrolimus. Results from RT-qPCR and ELISA performed in the present study indicate that the expression Th1-type cytokines, including IL-2 and IFN-γ, which have been reported to aggravate immune responses (31), were significantly inhibited by triptolide. These findings are in agreement with previous results (32). However, the Th2-type cytokine IL-10, which is essential for the induction of immune tolerance (33), was slightly increased in the cardiac grafts, whereas the peripheral blood levels were not affected, consistent with the results of a previous study on renal transplantation (34). These results suggest that the reduction of the expression and secretion of Th1-type cytokines are important mechanisms of triptolide that inhibit $CD4^+$ memory T cell-mediated acute rejection. Furthermore, the results of the present study demonstrate that the expression of TGF-β1 in the triptolide group was increased in the grafts and peripheral blood, in agreement with a previous study which demonstrated that triptolide may promote the induction of forkhead box $P3^+$ regulatory T cells (Tregs) via the upregulation of TGF-β1 (14). This indicates that inhibition of the Th1-type cytokine is not the only mechanism of action of triptolide, and that its effects on graft preservation may also be associated with the acceleration of Treg cell proliferation. However, $CD4^+$ memory T cell-mediated acute rejection is an extremely complex immune process.

The present study had certain limitations; for instance, it remains unknown whether the administration of triptolide as a monotherapy inhibits $CD4^+$ memory T cell-mediated acute rejection or induces immune tolerance completely. Previous studies have reported synergistic effects between triptolide and classic immunosuppressive agents, including rapamycin, cyclosporine A and FK506 (35-37), thus combined treatment may inhibit acute rejection more effectively than triptolide monotherapy. These results suggest that the combination treatment strategy, for example co-treatment with triptolide and rapamycin/FK506, may represent a potential treatment to prevent the rejection of grafted tissue or even induce immune tolerance.

In conclusion, the present study demonstrated that triptolide exerts a clear immunosuppressive effect on $CD4^+$ memory T cell-mediated acute rejection, and serves its inhibitory role via a number of possible mechanisms, such as inhibiting the secretion of Th1-type cytokines and promoting the proliferation of Tregs. However, future studies are required to clarify the mechanism of triptolide action.

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