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Statins induce immunosuppressive effect on heterotopic limb allografts in rat through inhibiting T cell activation and proliferation

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ABSTRACT

Long-term use of immunosuppressive agents could bring many side effects. Recently, 3-Hydroxy-3-methylgutaryl coenzyme A reductase inhibitors (statins) have been reported to be immunomodulatory besides lowering serum cholesterol level. The aim of this study was to investigate the effects of statins on composite tissue allografts and T lymphocyte in vivo and in vitro. Rats were divided into 5 groups: syngeneic transplantation group (Lewis–Lewis); allogeneic control group (Brown Norway–Lewis, no treatment); low-dose statins group (15 mg /kg); high-dose statins group (30 mg /kg) and cyclosporin A group. In vivo, treatment of statins significantly prolonged allografts survival as compared to control group. Histological findings further supported these clinical results and demonstrated less extent of rejection. Immunohistochemical analysis showed that there was a remarkably reduced T cells infiltration in statins groups. Moreover, the serum levels of IL-2 and IFN-γ were decreased after statins therapy, while these in control group increased significantly. Meanwhile, transcriptional activities of IL-2 and IFN-γ were also dramatically down-regulated after statins treatment. In vitro, mixed lymphocyte reaction assay was performed and the results revealed lymphocyte proliferation was inhibited by statins in a dose-dependent manner. Furthermore, administration of statins exhibited inhibitory effects on CD3/CD28 mediated T cell activation and proliferation. Besides, the results demonstrated that statins significantly down-regulated mRNA expression and suppress cytokine production of IL-2 and IFN-γ in vitro. In conclusion, our data demonstrated that application of statins could induce immunosuppressive effect and prolong allografts survival through inhibiting activation and proliferation of T cell and reducing production of IL-2 and IFN-γ.

1. Introduction

Clinical composite tissue transplantation has been introduced as a new approach for reconstruction in plastic surgery (Cendales et al., 2005b; Lanzetta et al., 2005; Levi et al., 2003; Margreiter et al., 2002). However, rejection is still a leading cause of failure after transplantation. Despite enormous developments in immunosuppressant for improving allografts survival, patients must depend on therapy for life and may suffer severe side effects including increased risk of infections and certain cancers. As a result, these obstacles provide the impetus for the development of new agents for allograft recipients.

Recently, it has been demonstrated that the 3-Hydroxy-3-methylgutaryl coenzyme A (HMG-CoA) reductase inhibitors or statins play a multifactorial role in the maintenance of transplanted organs (Blanco-Colio et al., 2003; Raggatt and Partridge, 2002; Suzuki et al., 2007). As clinical outcomes become available, it appears that statins are proved to be more beneficial than expected and the actions of statins go beyond their ability of lowering down serum cholesterol levels (Kobashigawa et al., 2005; Weis et al., 2001). Previous studies had reported that statins decreased the number and severity of acute rejection episodes after organ transplantation (Kakkis et al., 1997; Keogh et al., 2000). Until now, the cellular actions of statins which have been identified mainly include two mechanisms by which statins can potentially modulate functional maturation of T lymphocytes. Firstly, statins regulate inducible class II major histocompatibility complex (MHC) expression on macrophages and endothelial cells (Kwak et al., 2000). Secondly, statins modulate T-cell costimulation through direct effects on leukocyte function antigen-1 (LFA-1)/intercellular adhesion molecule-1 (ICAM-1) interactions, dependent upon recognition of a novel statins binding site on beta2 integrins (Weitz-Schmidt et al., 2001). Therefore in the present study, we focused on T lymphocytes, investigated the inhibitory effect of statins on T cells in vivo and in vitro and explored its potential immunosuppressive function.

Effective activation of T cell requires two signals: T cell receptor engagement by a peptide/MHC complex and costimulation signal.

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After recognizing the antigen peptide presented on the surface of antigen-presenting cell, naive T cells differentiate into effector cells and respond vigorously with clone expansion. Upon T cell activation and proliferation, IL-2 and IFN-γ produced by T cells exert powerful positive immunoregulatory function (Pae et al., 2004; Tewari et al., 2007). In contrast, IL-10 is another cytokine with negative regulation considered as a potent inhibitor of antigen-presenting cells and T-lymphocyte functions (Mocellin et al., 2003). Since the immuosuppressive effect of statins had been considered mainly towards T cells, we propose that statins might be correlated with cytokine production including IL-2, IFN-γ and IL-10.

Statins are currently being assessed for application of immunosuppression which prevents rejection of organ transplantations (Steffens and Mach, 2006). However, immunological aspects of the statins treatment on composite tissue allografts are still obscure. Composite tissue allografts differ from solid organ transplants, as they contain different types of tissues, including skin, subcutaneous fat, muscle, tendon, cartilage, bone, bone marrow, vessels, and nerves. We had successfully established a heterotopic limb transplantation model in rat to evaluate immunological effect of statins. Due to the potential role of statins as a regulator of immune system, we hypothesize that statins would delay the occurrence of rejection and prolong allograft survival after transplantation.

Thus in this study, to find whether statins achieved immunosuppressive effect through inhibiting T cell activation and proliferation, we investigated the effects of statins on composite tissue allografts and T lymphocyte in vivo and in vitro. Statins with potent immunomodulatory effect might be applied as a novel therapeutic approach for suppression of rejection.

2. Materials and methods

2.1. Animals and treatment

Male Lewis rats and Brown Norway rats (200–250 g) were provided by the Vital River laboratory animal company (Beijing, China). All animals received humane care in compliance with "The Principles of Laboratory Animal Care" formulated by the National Society of Medical Research and "The Guide for the Care and Use of Laboratory Animals" published by the U.S. National Institutes of Health. The following experimental protocol was approved by local ethical committee.

The animals were assigned randomly into five groups and performed heterotopic limb transplantations (n=8 for each group). Group 1: Lewis-to-Lewis syngeneic transplantation group; Group 2: Brown Norway-to-Lewis allogeneic control group receiving no immunosuppression; Group 3: low-dose atorvastatin treated group (15 mg/kg/day); Group 4: high-dose atorvastatin treated group (30 mg/kg/day); Group 5: Cyclosporin A treated group (16 mg/kg/day) (Demir et al., 2004). All surgical procedures were performed under sterile conditions. Anesthesia was induced with sodium pentobarbital (30 mg/kg, administered intravenously). Hair on the abdominal and right hind limb was shaved and the skin was cleansed with povidone-iodine (10%) solution. Postoperatively, the recipients received Rocephin (20 mg/kg/day, Roche, Switzerland) for 7 days Atorvastatin (Pfizer, Dalian, China) and cyclosporin A (Sandimmun, Sandoz, Basel, Switzerland) were dissolved in phosphate-buffered saline solution and orally administered daily.

2.2. Surgical technique

Donor operation: The surgical procedure was performed similarly as previously described (Nazzal et al., 2004). An oblique incision was performed in the inguinal region. The femoral vessels were isolated and prepared. The epigastric skin flap, approximate 4×5 cm, was harvested based on the epigastric artery and vein. The limb was transected at the ankle joint. Subsequently, the abductor muscle groups were separated from their insertions in the third trochanter. The adductor muscles were gently dissected to protect the muscular branch of the femoral artery. The medial and lateral circumflex arteries, which are branches of the hypogastric artery, nourish the femoral neck and head. Since they arise from the hypogastric artery, they cannot be preserved. Thus, following separation of the gluteus medius, gluteus minimus, and piriformis muscles from their insertion, the femur was transected at the level of the lesser trochanter. The femoral vessels, which supply the femoral shaft and the attaching muscles as well as the skin flap, were carefully preserved.

Recipient operation: A 4×5 cm defect was created in the anterior abdominal and medial thigh region of the recipient to accommodate the donor skin flap. The femoral vessels of recipient were dissected and prepared for anastomosis. The limb allograft was simply placed in the inguinal area without fixation, and covered with the vascularized flap. Standard end-to-side microsurgical anastomosis was performed between donor and recipient's femoral vessels using 10/0 nylon. Skin was sutured with 4/0 absorbable suture. The animals were put under a heating lamp till full recovery from anesthesia.

2.3. Clinical evaluation for allografts

Transplanted allografts were evaluated on a daily basis for any signs of vascular failure. After heterotopic limb transplantation, vena caudalis blood was taken for biochemical analysis at 7 day, including serum cholesterol, and triglyceride. In the early postoperative period, skin flaps were observed for color and temperature changes, hematoma formation, progressive edema, and later for signs of rejection including erythema, edema, loss of hair, desquamation and ulceration.

2.4. Histological analysis

The samples of the skin grafts were done at posttransplant day 7, 14, and 21 on routine hematoxylin-and-eosin stained sections. Histological rejection was graded according to previously published criteria (Table 1) (Cendales et al., 2005a). Five skin biopsies were taken from the allografts in each group for histopathological evaluation. Finally, histological results were scored which is consistent with the grade of rejection and expressed as mean±S.E.M. In addition, biopsies were taken at the presence of clinical signs of rejection for assessment of the degree of rejection.

2.5. Immunohistochemistry analysis

Biopsies were embedded with OCT and frozen in liquid nitrogen immediately for immunostaining. Frozen sections (6 um) were incubated with primary monoclonal antibodies (mAb) against CD8 (1:200 dilution, mouse anti rat CD8, MCA48GA, AbD Serotec) and CD4 (1:200 dilution, mouse anti rat CD4, MCA153GA, AbD Serotec) T cell respectively. They were then incubated with secondary antibody and antigen–antibody conjugates were detected with peroxidase anti-peroxidase complex (goat anti mouse, Zhongshan Goldenbridge Biotechnology Company, Beijing, China) according to the manufacturer's instructions. Positively stained cells per high power field were counted in ten random fields, and then the counts were averaged.

Table 1
Histopathological grading system for rejection reaction

	Epidermis	Dermis
Grade 0	Normal	Normal
Grade 1	Focal mononuclear cell infiltration and cell vacuolation	Mild lymphocytes increase
Grade 2	Suprabasal bulla formation	Moderate mixed cell infiltration
Grade 3	Necrosis	Vasculitis, thrombosis, severe rejection

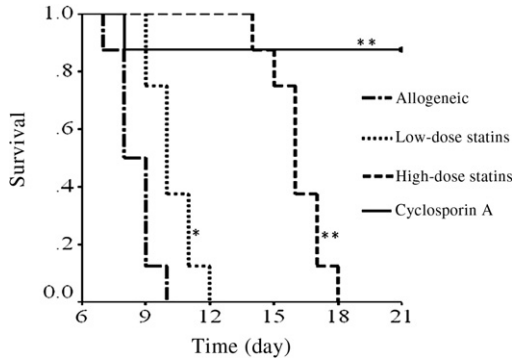

Fig. 1. Statins prolonged heterotopic limb allografts survival. Recipients in low-dose or high-dose statins groups showed prolonged limb allografts survival compared with allogeneic control group. Data are shown as the mean±S.E.M of the results from five separate experiments. *$P<0.05$, **$P<0.01$ versus allogeneic control group.

2.6. Enzyme-linked immunosorbent assay (ELISA)

The secretion of interleukin-2 (IL-2, 1:200 dilution), interferon-γ (IFN-γ, 1:200 dilution), and interleukin-10 (IL-10, 1:200 dilution) (R&D System, Minneapolis, MN, USA) were detected with ELISA Kits specific for the rat cytokines. The serum samples were collected at posttransplant day 3, 7 and 10. We have performed Enzyme-linked Immunosorbent assays strictly according to the manufacturer's instruction. The optical density was determined by Spectrophotometer under the 450 nm wave length and the concentrations of IL-2, IFN-γ and IL-10 were calculated.

2.7. Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Semiquantification of the mRNA expression of IL-2, IL-10 and IFN-γ was performed by RT-PCR method. Total RNA was extracted from the allografts at posttransplant day 7 with Trizol reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. A reverse transcription reaction was performed with 1 μg total RNA using reverse transcriptase from RNA PCR Core kit (Roche) under standard conditions. Primers sequences for each gene were as follows: for IL-2 (sense, 5′-CCC CAT GAT GCT CAC GTT TA-3′; antisense, 5′-ATT TTC CAG GCA CTG AAG ATG TTT-3′), IL-10 (sense, 5′-GAA TCA GCA GCG ACT CCT TT -3′; antisense, 5′-TTG CTG ATG GCC TGA TTG TC-3′), IFN-γ (sense, 5′-AGT CTG AAG AAC TAT TTT AAC TCA AGT AGC AT-3′; antisense, 5′-CTG GCT CTC AAG TAT TTT CGT GTT AC-3′), β-actin (sense, 5′-CCT TCC TGG GTA TGG AAT CCT-3′; antisense, 5′-GGA GCA ATG ATC TTG ATC TT-3′). cDNA templates were amplified as follows: 5 min at 95 °C, followed by 30–35 cycle s of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min. At the end of the cycling, the samples were incubated at 72 °C for 5 min. The amplified DNA products were separated on an 8% polyacrylamide gel and stained with ethidium bromide. The intensities were determined with a fluoroimager (FluorImager 595, Amersham Biosciences). Each sample was analyzed in triplicate. Results were obtained and normalized to β-actin expression which was used as endogenous control.

2.8. Mixed lymphocyte reaction

Mixed lymphocyte reaction assay was performed with responder splenocytes from Lewis rat and mitomycin-C (Sigma, St. Louis, MO) inactivated stimulator splenocytes from Brown Norway rat. The lymphocyte proliferation in response to alloantigen was determined by [^{3}H]-thymidine incorporation. Briefly, a total of 2×10^5 responder cells and an equal number of stimulator cells were co-cultured in 96-well culture plates at 37 °C under 5% carbon dioxide for 72 h. Atorvastatin was added to each well at various concentrations on day 0. Then they were pulsed with 1 μCi/well [^{3}H] thymidine (China Isotope Corporation, China) for 18 to 24 h before harvest and assessed by a beta counter. Cell proliferation is expressed as the counts per minute (cpm) of the responder cells.

Table 2
Total cholesterol and triglyceride at posttransplant day 7

Group/(n=8)	Syngeneic	Allogeneic	Low statins	High statins	Cyclosporin A
Total cholesterol	128±17	119±16	101±9[a]	88±12[a]	121±13
Total triglyceride	173±20	178±18	162±12	159±14	169±16

[a]$P<0.05$ vs syngenic group (mg/dl).

2.9. CD3/CD28 costimulation induced primary T cell activation

Primary T cells (2×10^5/well) purified from lymph node of normal Lewis rat were co-cultured with anti-CD3 (4 μg/ml) and anti-CD28 (2 μg/ml) monoclonal antibodies (BD Biosciences, San Diego, CA, USA) in 96-well culture plates. Statins at indicated concentrations were added to each well at 37 °C under 5% carbon dioxide for 48 h. Cells were pulsed with 0.5 μCi/well [^{3}H]-thymidine for 8 h before the termination of cultures and assessed for [^{3}H]-thymidine uptake.

To investigate mRNA expression of IL-2, IFN-γ and IL-10, total cellular RNA was extracted from primary T cells with Trizol reagent (Invitrogen) after stimulation with anti-CD3 and anti-CD28 for 6 h in

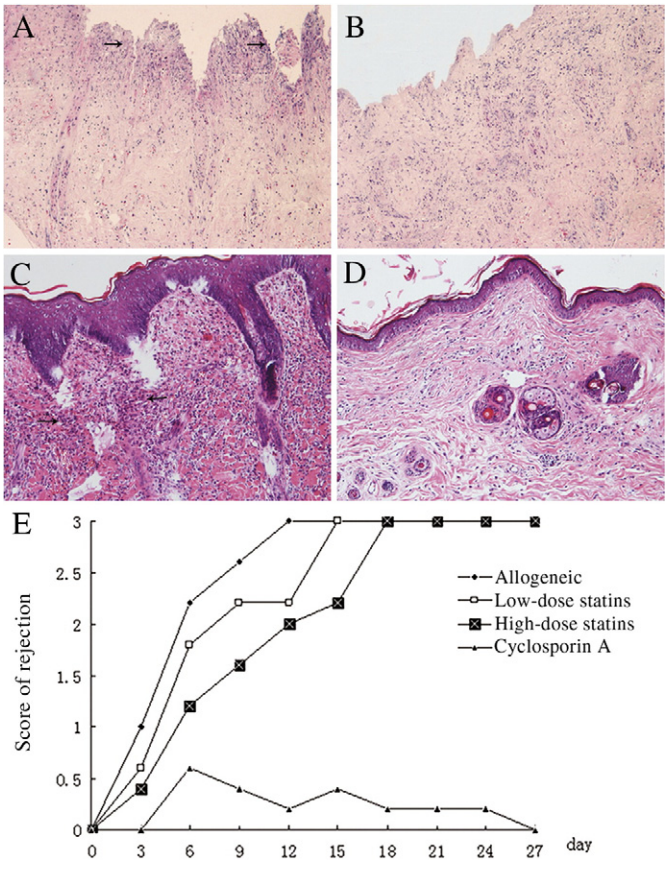

Fig. 2. Histologic evaluation of allografts at posttransplant day 7. In the allogeneic control group (A), skin samples showed necrotic epidermis feature (arrows) of grade III rejection reaction. (B) Skin biopsies in low-dose statins group revealed typical pathological changes of grade II rejection. (C) The biopsy samples taken from high-dose group revealed mild to moderate lymphocyte infiltration (arrows), while biopsies from cyclosporin A group showed normal histology (D). Original magnification 100×. (E) The score of rejection was significantly decreased in high-dose statins group as compared to control group ($P<0.05$), while there was no difference between low-dose group and control group.

the absence or presence of statins. cDNA was performed under standard conditions using reverse transcriptase from RNA PCR Core kit (Roche). The following procedures of RT-PCR were consistent with the described above. The relative gene expression was normalized to β-actin expression for each sample. Moreover, to determine cytokine levels, culture supernatants were harvested at 24 h to measure IL-2, IFN-γ and IL-10 levels by ELISA according to the manufacturer's instruction.

2.10. Statistical analysis

All data are expressed as mean±S.E.M. The Mantel log-rank test was used for comparison of allografts survival. Comparisons between the groups were performed using either student's *t*-test or one-way analysis of variance (ANOVA) followed by Dunnett's test. $P<0.05$ was considered statistically significant.

3. Results

3.1. Effect of statins on heterotopic limb allografts survival in rat

We did not observe vascular problems in any of the allografts. In syngeneic group, all recipients survived indefinitely. In allogeneic control group, hind limb rejection occurred on average on day 8.37±0.93. In low-dose statins group, mean survival time of allografts was 10.25±1.03 days. In high-dose statins group, mean survival time of allografts was 16.13±1.24 days. The data exhibited that treatment of statins remarkably achieved immunosuppressive effects and significantly prolonged allografts survival. There was marked difference in high-dose or low-dose statins group as compared with control group ($P<0.05$) (Fig. 1). Recipients in cyclosporin A group showed normal appearance without signs of rejection until the end-point. However, one recipient died as a result of anorexia and poor general condition on day 5. In addition, there was no difference in plasma triglyceride level between the statins treated groups and syngeneic group, while total cholesterol level was decreased significantly in statins group (Table 2).

3.2. Effect of statins on histological changes of allografts

The histological outcomes were well correlated with the macroscopic appearance. In control group (Fig. 2A), skin samples showed typical features of grade III rejection at day 7 including necrosis of the epidermis and severe lymphocytes infiltrate, while in low-dose statins group appeared less extent of rejection (Fig. 2B). Biopsies taken from high-dose statins group revealed mild to modest mixed cell infiltrate in the dermis at day 7 (Fig. 2C). In contrast, histologic examination revealed that acute rejection was suppressed in allografts treated with cyclosporin A and showed normal-appearing epidermis, dermis, and adnexa (Fig. 2D). The score of rejection showed that treatment of high-dose statins significant decreased the extent of rejection and

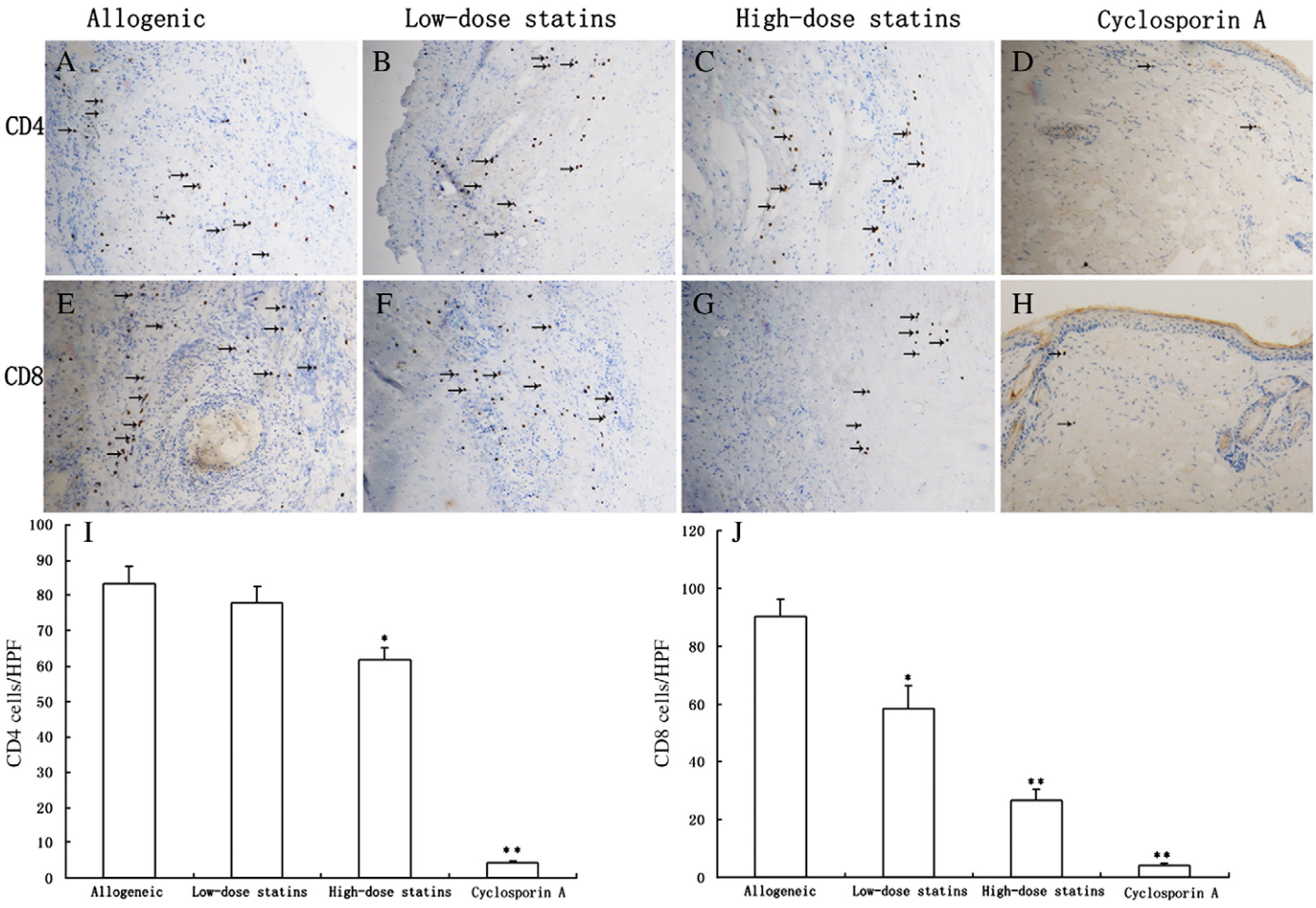

Fig. 3. Immunohistochemical analysis of $CD4^+$ and $CD8^+$ T cells in skin biopsy specimens of allografts. Immunohistochemical staining showed that CD4 and CD8 positive T cells stained in brown (arrows) were remarkably expressed in allogeneic control group at posttransplant day 7 (A, E). There was less T lymphocyte infiltration in low-dose statins group (B, F). In high-dose statins group (C, G) and cyclosporin A group (D, H), the extent of stain for $CD4^+$ and $CD8^+$ T cells respectively was significantly attenuated compared with control group. Original magnification 100×. The number of $CD4^+$ T cells was remarkably reduced in high-dose statins group (I), while the number of $CD8^+$ T cells was also decreased significantly in both low-dose and high-dose statins groups. (J). Data are shown as the mean±S.E.M of the results from three samples. *$P<0.05$, **$P<0.01$ versus allogeneic control group.

prolonged allografts survival. However, there were significant differences between statins groups and cyclosporin A group (Fig. 2E).

3.3. Effect of statins on $CD4^+$ and $CD8^+$ T cells infiltration of allografts

There were less CD4 and CD8 positive T cells in statins treated group at day 7 after transplantation. Immunohistochemical staining showed that positive cells were nearly absent in cyclosporin A group but were remarkably expressed in allogeneic control group (Fig. 3A–H). The outcome was consistent with previous histological findings. There was significant difference of $CD4^+$ T cells only in high-dose statins group as compared with control group ($P<0.05$) (Fig. 3I). The infiltration of $CD8^+$ T cells was significantly attenuated in both low-dose and high-dose statins treated group (Fig. 3J). In addition, $CD8^+$ T cells in high-dose statins group decreased evidently in comparison with $CD4^+$ T cells. The results suggested that the immunosuppressive effect of statins was performed mainly towards T cells in vivo.

3.4. Effect of statins on the serum levels and mRNA expression of IL-2, IL-10 and IFN-γ in vivo

Because of the mainly effect of statins on T cells, we further investigated the production of IL-2, IFN-γ and IL-10, which were produced by T cells and exerted powerful immunoregulatory function. The serum levels of IL-2 and IFN-γ were markedly elevated after the allotransplant procedure in allogeneic control group at posttransplant day 3, 7 and 10 (Fig. 4A, B). Statins treated therapy yielded a significant down-regulation of these cytokines compared with control group, which were also found in cyclosporin A group (Fig. 4A, B). There was no difference in IL-10 between statins treated group and control group. Interestingly, we observed that IL-10 level mildly decreased in cyclosporin A treated group at day 10 (Fig. 4C).

By reverse transcription-PCR assays, compared with allogeneic control group, mRNA expression of IL-2 and IFN-γ also dramatically reduced after statins treatment at posttransplant day 7 (Fig. 5A, B). The impaired cytokine mRNA expression paralleled with the decreased cytokine production detected in serum, suggesting that statins affected gene expression upon T cell proliferation. There was no difference in mRNA expression of IL-10 between the groups (Fig. 5C).

3.5. Effect of statins on mixed lymphocyte reaction

To test the effect of statins in various concentrations for lymphocyte proliferation in vitro, mixed lymphocyte reaction was performed using responder splenocytes cells from Lewis rat and inactivated stimulator splenocytes from Brown Norway rat. We did not observe inhibitory effect of low-concentration atorvastatin on alloantigen-induced proliferation of lymphocyte. However, lymphocyte proliferation was suppressed by the treatment of high-concentration atorvastatin in a dose-dependent manner (Fig. 6). There was significant difference compared with blank control group ($P<0.05$). The results indicated that statins had remarkable inhibitory effect on lymphocyte proliferation.

3.6. Effect of statins on CD3 plus CD28 mediated primary T cell responses in vitro

To further examined the direct effect of statins on the activation and proliferation of T cells in vitro, we purified primary T cells from lymph node of normal Lewis rat and induced effective T cell activation through CD3 and CD28 costimulatory signals. The results presented in Fig. 7A revealed inhibitory effect of statins on CD3 plus CD28 mediated primary T cell responses. The use of anti-CD3 and anti-CD28 agonistic antibodies induced a remarkable proliferation of T cells and up-regulated mRNA expression of IL-2 and IFN-γ. However, treatment of statins could significantly down-regulate their expression presented in RT-PCR results (Fig. 7B). These might be correlated with the immunosuppressive effects of statins on T cells. Moreover, as showed in Fig. 7C, administration of statins also profoundly suppressed cytokine production including IL-2 and IFN-γ. The decreased cytokine

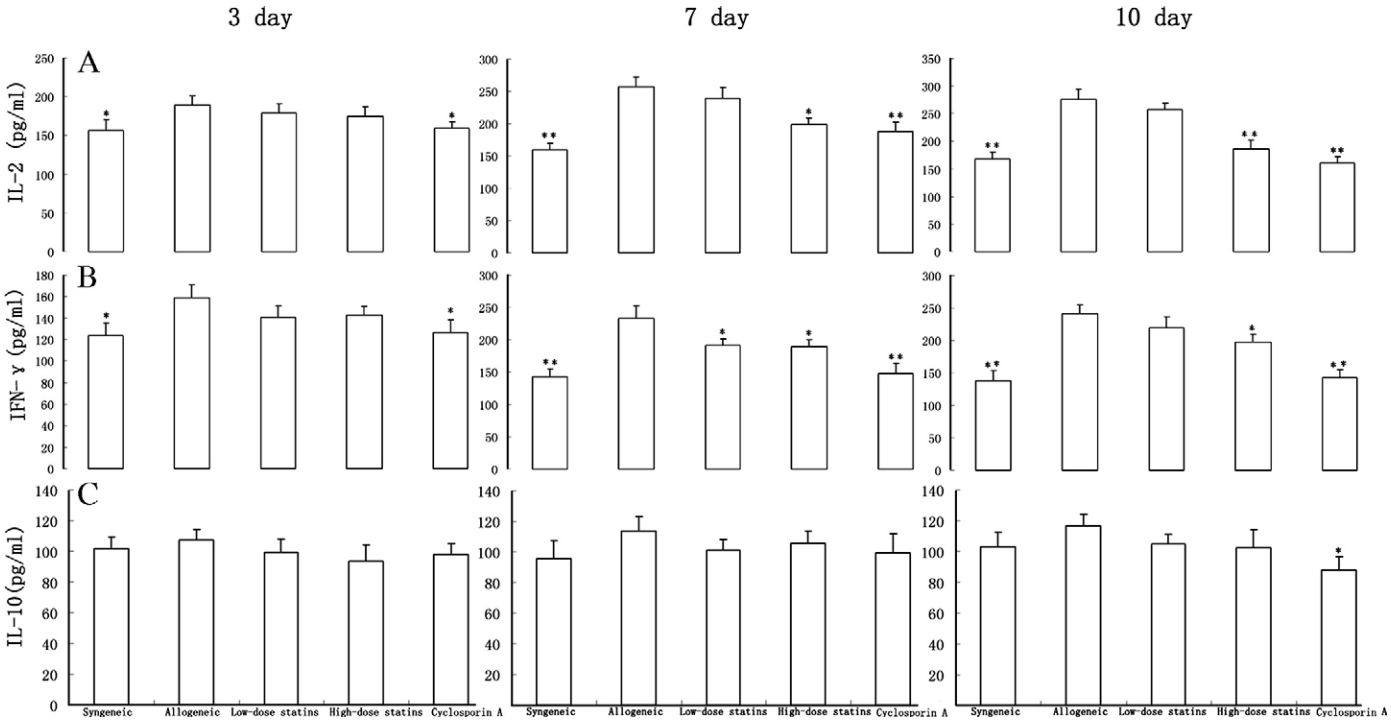

Fig. 4. The serum levels of IL-2, IFN-γ and IL-10 after transplantation. The levels of both IL-2 (A) and IFN-γ (B) were markedly elevated in allogeneic group, especially at posttransplant day 7 and 10, while the levels were decreased in statins or cyclosporin A group. There was no difference of IL-10 between statins group and control group (C). Data are shown as the mean ± S.E.M of the results from three samples. $*P<0.05$, $**P<0.01$ versus allogeneic control group.

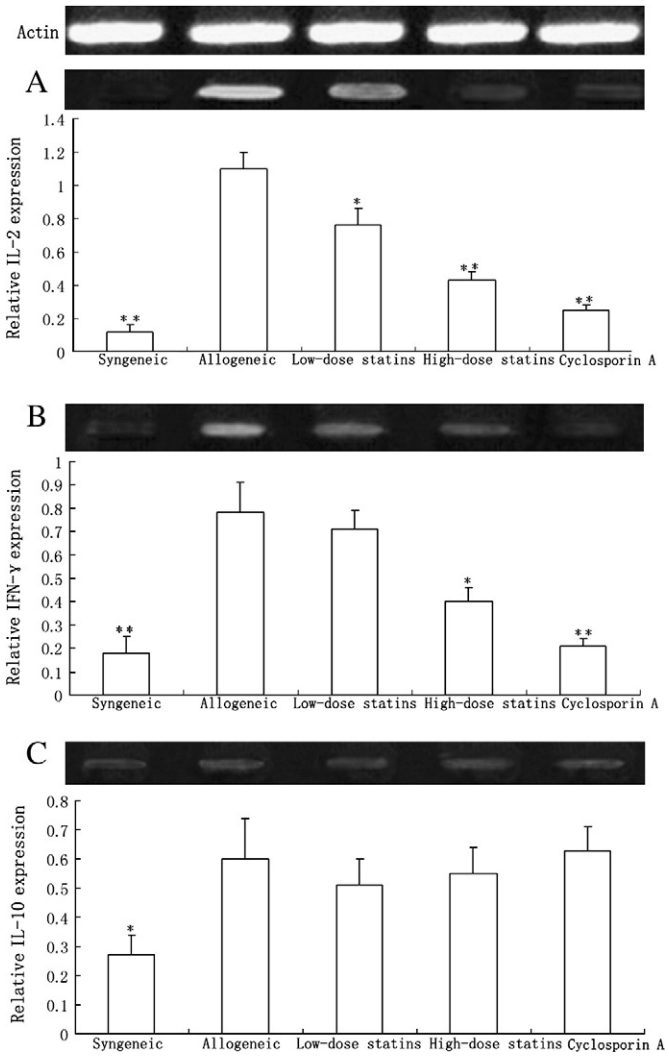

Fig. 5. The mRNA expression of IL-2, IFN-γ and IL-10 in allografts. Electrophoresis images of IL-2, IFN-γ and IL-10 mRNA levels from five groups were presented at posttransplant day 7. The semi-quantification results of RT-PCR exhibited that mRNA expression of IL-2 (A) and IFN-γ (B) was down-regulated after statins or cyclosporin A therapy, while the level of IL-10 (C) was not influenced by drugs treatment. Data are shown as the mean±S.E.M of the results from three samples. *$P<0.05$, **$P<0.01$ versus allogeneic control group.

production detected in culture supernatants paralleled with the impaired mRNA expression of cytokine. We observed there was no difference in IL-10 level between statins treatment and non-treatment groups. These results demonstrated that statins directly inhibited CD3/CD28 mediated T cell responses in vitro.

4. Discussion

Long-term immunosuppression is our major goal in both allogeneic organ transplantation and composite tissue transplantation. The development of powerful immunosuppressive agents, such as cyclosporin, has lowered the incidence of acute rejection episodes. However, long-term use of these agents could bring many side effects that may be potentially dangerous in future. Therefore, there is a need of searching for new potent immunosuppressive agents with minimal toxicities. Recently, statins have been reported to not only lower serum cholesterol level but also have immunomodulatory effects (Keogh et al., 2000). It had been reported that statins substantially suppress endothelial cell MHC-II expression in vivo and inhibit organ-specific immune responses (Geissler et al., 2006). In addition, an immunosuppressive effect of statins in organ transplantation had been indicated (Kobashigawa, 2004). In this study, we investigated the effects of statins on composite tissue allografts and T lymphocyte in vivo and in vitro study respectively.

First, we successfully established a heterotopic limb allotransplantation model in rat to observe immunosuppressive effects of statins in vivo. Our results revealed that statins therapy significantly prolongs allografts survival, especially high-dose statins treatment. As showed in Table 2, there was no difference in plasma triglyceride level between the statins treated groups and syngeneic group. This indicated that statins administration did not influence plasma triglyceride level in normal individual. Moreover, the histological outcome demonstrated a significant immunosuppressive effect of statins and showed that there was less inflammatory cell infiltration in statins treated groups. The score of rejection revealed that treatment of statins significant decreased the extent of rejection and prolonged allografts survival. In addition, immunohistochemical staining further confirmed that there was an elevated level of T cell infiltration on allografts in control group. However, as counted as CD4 and CD8 positive cells respectively, the number of infiltrating cells was significantly reduced in statins group as compared with control group. Although the mechanisms by which statins achieved immunosuppressive function are not fully understood till now, it is reasonable to believe that the direct effect of statins might be mainly towards T cells. Our studies confirmed that treatment of statins can reduce T lymphocytes infiltration in vivo. The data also suggested that far more $CD8^+$ T cells than $CD4^+$ T cells were reduced by statins treatment on allografts. This may be correlated with the changes of cytokines including IL-2, IFN-γ and IL-10 (Boleslawski et al., 2004; Horwitz et al., 2003; Tewari et al., 2007; Wong and Pamer, 2004). The above results suggested that administration of statins could reduce T lymphocyte infiltration and prolong allografts survival in vivo.

The abundance of lymphocyte infilitration would be related to the levels of locally produced cytokines and chemokines, which stimulate and promote the inflammation process after transplantation. The previous reports had indicated the expression of cytokines including IL-2, IFN-γ and IL-10, and chemokines were all reduced by statins treatment (Arnaud et al., 2005; Kwak et al., 2000). These were consistent with our results of the levels of IL-2 and IFN-γ. In the present study, we found that both serum level and mRNA expression of IL-2 were significantly inhibited after statins treatment on allografts. High-dose statins therapy exhibited stronger inhibitory effect than low-dose statins therapy. The similar phenomemon as observed in IL-2 was also found in IFN-γ expression. Previous studies (Bishop et al., 2002; Qian et al., 1997; Sharland et al., 1998)

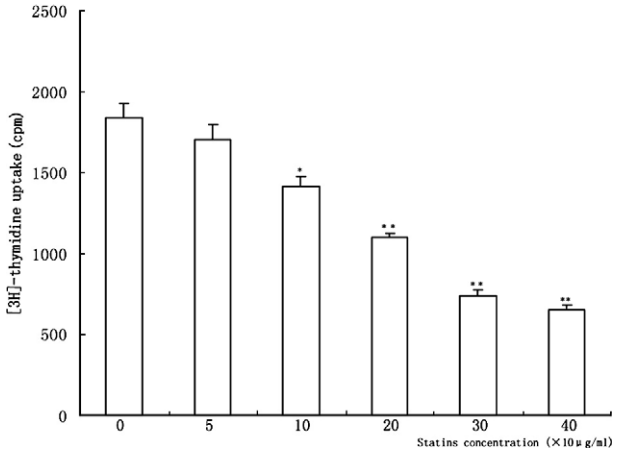

Fig. 6. Statins inhibited mixed lymphocyte reaction. The result illustrated that atorvastatin inhibited lymphocyte proliferation in dose-dependent manner. Data are shown as the mean±S.E.M of the results from five separate experiments. *$P<0.05$, **$P<0.01$ versus blank control group.

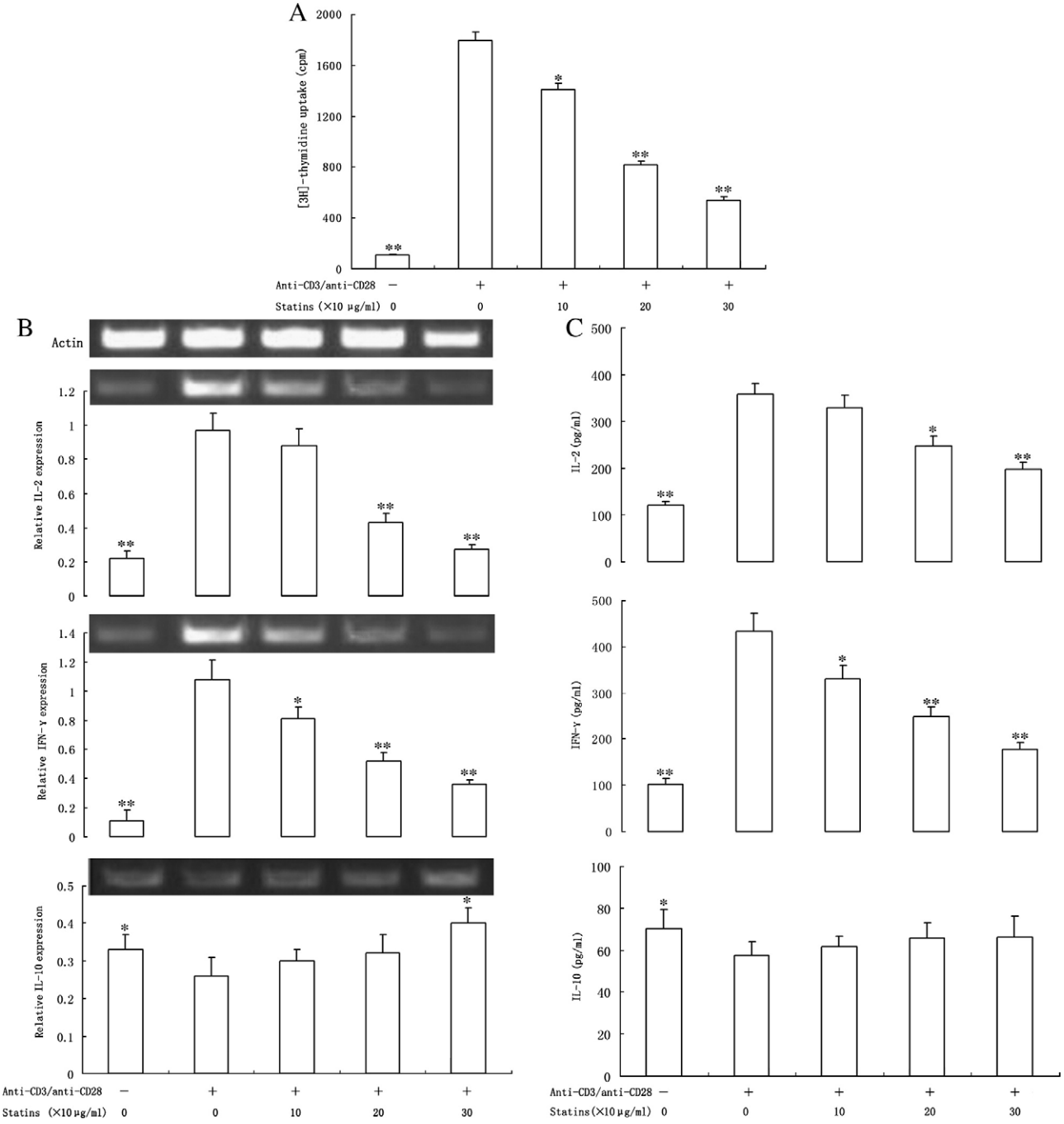

Fig. 7. Statins inhibited CD3 plus CD28 mediated primary T cell proliferation and cytokine production in vitro. Primary T cells from lymph nodes were cocultured with anti-CD28 and anti-CD3 mAb in the absence or presence of statins at indicated concentrations for 48 h. The results showed that statins inhibited CD3/CD28 mediated T cell responses (A). Cytokine expression at the mRNA level and production at the protein level were determined after 6 h and 24 h of stimulation respectively. CD3/CD28 costimulation induced a remarkable mRNA expression (B) and cytokine production (C) of IL-2 and IFN-γ. However, treatment of statins could significantly down-regulate their mRNA expression and suppress cytokine production. There was no difference in IL-10 level between statins treatment and control groups. Data are shown as the mean ± S.E.M of the results from three separate experiments. *$P<0.05$, **$P<0.01$ versus non-treatment control group.

demonstrated that inhibition of IL-2 and IFN-γ expression significantly reduced T lymphocytes activation and proliferation, thus elevated allografts survival. In this study, we proposed that statins prolong allografts survival in part by decreasing the expression of IL-2 and IFN-γ. IL-10 is a potent stimulator of B-lymphocytes as well as a powerful inhibitor of antigen-presenting cells and T-lymphocyte functions (Mocellin et al., 2003). However, it was reported that IL-10 enhances $CD8^+$ T cells primary responses and inhibits secondary responses (Kang and Allen, 2005). Besides, as a potent inhibitor of IL-10 production, tacrolimus treatment demonstrated a better graft survival in many clinical trials (Jiang et al., 2001). In fact, the underlying effect of IL-10 on allografts is still controversy and this might be linked to the complexity of its dual effects (Mocellin et al., 2003). Our study showed that there was no difference of IL-10 expression between statins treated and control groups.

Since the in vivo immunosuppressive effects of statins had been considered mainly towards T cells, we performed mixed lymphocyte reaction to examine the effect of statins blockade on lymphocyte proliferation in vitro study. Mixed lymphocyte reaction is a model of T-cell response to alloantigenic peptides complexed with major

histocompatibility proteins on antigen-presenting cell. The result showed that T cell proliferation was reduced evidently in a dose-dependent manner. We also found there was difference between high-dose and low-dose statins group in vivo study. This indicated the proper dosage of statins should be recommended for evaluation of its oral safety and immunosuppressive effect with high efficiency (Kobashigawa, 2004; Sabbatini et al., 2004). To further examine the direct effect of statins on the activation and proliferation of T cells in vitro, we induced effective T cell activation in the presence of CD3 and CD28 costimulatory signals. The costimulation of T cells in vitro via agonistic antibodies to T cell receptor was performed to mimic T cell activation in the physiological conditions. The use of anti-CD3 plus anti-CD28 mAb induced a remarkable proliferation of T cells and cytokine production of IL-2 and IFN-γ that are Th1-type cytokines. The results revealed that statins concentration-dependently inhibited CD3/CD28 mediated T cell responses. In addition, compared with control group, treatment of statins significantly down-regulated mRNA expression and suppress cytokine production of IL-2 and IFN-γ. Despite the inhibitory effect of statins on Th1-type cytokine production, we did not observe the similar effect on Th2-type cytokine (IL-10) that was consistent with the in vivo study. IL-2 and IFN-γ have been implicated in the pathogenesis of Th1-mediated immunological diseases (Ruschpler and Stiehl, 2002). The data indicated statins could delay the occurrence of rejection after transplantation by suppressing Th1-type immune responses but not Th2-type responses. Compared with IL-10, IL-2 and IFN-γ play an important role in statins-mediated immunosuppressive effect. These results further demonstrated that statins inhibited T cell activation and proliferation and prevented production of IL-2 and IFN-γ in vitro.

The use of statins had improved long-term outcome after transplantation, with prolongation of hind limb survival. However, all allografts except for cyclosporin A treated group were finally rejected, suggesting inhibition of statins alone cannot induce completely donor-specific tolerance. Compared with cyclosporin A group, there still existed significant difference of T cell counts and allografts survival in statins group. Although the immunosuppressive effect was moderate, it was reasonable to believe that statins could decrease the levels of IL-2 and IFN-γ and inhibit T cell activation and proliferation, thus delay the occurrence of allografts rejection. In future, we may further determine whether the combination therapy with conventional immunosuppressant will be more feasible and desirable than monotherapy in composite tissue transplantation. In addition, the best way to administer and long-term efficacy of statins should also be taken into consideration before clinical application.

In conclusion, this study reported the immunosuppressive effects of statins both in vivo and in vitro study. Our finding rationally demonstrated that treatment of statins significantly prolonged allografts survival in a dose-dependent manner through inhibiting activation and proliferation of T cell and reducing cytokine production of IL-2 and IFN-γ. The experimental data provided important evidence that statins have a potential to regulate immune response in vivo and in vitro and therefore might be of particular value for the suppression of rejection.

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