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Obaculactone suppresses Th1 effector cell function through down-regulation of T-bet and prolongs skin graft survival in mice

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ABSTRACT

Allograft rejection is a predominantly Th1 immune response. In this study, we showed that obaculactone, a natural compound derived from citrus fruit, prolonged skin graft survival in mice when treated after but not before transplantation. Furthermore, obaculactone inhibited alloantigen-specific production of Th1 cytokine IFN-γ as well as proinflammatory cytokine IL-2, TNFα and IL-6. In parallel, IL-10 production was markedly up-regulated. Obaculactone significantly enhanced the percentage of $CD4^+CD25^+Foxp3^+$ Treg cells in the $CD4^+$ splenocytes without any effect on their inhibitory function. *In vitro* and *in vivo* tests showed obaculactone down-regulated T-bet expression in Th1 effector cells. Taken together, the unique immunomodulatory properties might qualify obaculactone as a putative, therapeutic compound for the treatment of Th1-driven diseases, including transplant rejection.

1. Introduction

CD4 Th cells play a central role in the transplantation immunity [1]. An allograft is rejected by intragraft immune responses, including infiltration of alloreactive T cells into the graft and the activation of these cells by Th1-associated cytokines [2]. It has been proposed that a Th1 phenotype can promote allograft rejection via IFN-γ-induced activation of macrophage function. In contrast, the Th2 phenotype has been proposed to favor longer graft survival in some models [3]. Therefore, the hallmark Th1 cytokine IFN-γ is frequently used in the determination of the functional status of alloreactive T cells during the development of transplantation rejection [2,4,5]. Recently, the critical role of IL-17 in alloimmune responses adds an additional dimension to this complex network involving a battery of cytokines [3].

In order to prevent allograft rejection, most current immunosuppressive drugs nonspecifically target T-cell activation, clonal expansion or differentiation into effector cells. Despite their efficacy, the drugs carry the risks of adverse effects including kidney failure, cancer and infections. Calcineurin inhibitors such as cyclosporine A (CsA) which mainly inhibit CD4 Th cells are now importantly positioned as the cornerstone of immunosuppressive therapy in organ transplantation. Nevertheless, continual treatment has a significant adverse impact on renal function and cardiovascular disease, and extended long-term graft survival has not been achieved. Previous studies indicate that CsA is ineffective in inhibiting effector T cells while it can prevent naïve T cell activation [5]. The treatment is generally started before or at the time of transplantation with the goal of preventing acute rejection [6]. We hypothesized that it might be a more attractive strategy to selectively inhibit the functional response of pathogenic effector T cells to the allograft without damaging the normal immunocompetence.

Obaculactone (7,16-dioxo-7,16-dideoxylimondiol), also called limonin, is a triterpenoid dilactone extracted from citrus fruit. *In vivo*-tests have shown that obaculactone inhibits carcinogen-induced tumor growth in different organs [7,8]. In addition to anticancer properties, obaculactone shows anti-inflammatory activity by eliciting a suppressive effect on $CD4^+$ T cells [9]. Moreover, obaculactone down-regulates inducible nitric oxide synthase and cyclooxygenase-2, inhibits NF-κB p65 nuclear translocation, and is proposed capable of modulating $CD4^+$ T-cell function [9,10]. To address our hypothesis, we compared the effects on skin allograft rejection of obaculactone treatment after transplantation with those before transplantation. The results revealed obaculactone as a potential immunomodulatory agent which down-regulated the action of IFN-γ in Th1 effector T cells and up-regulated the frequency of $CD4^+CD25^+Foxp3^+$ Treg cells during the ongoing alloresponses.

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2. Materials and methods

2.1. Animals and agents

Male Balb/c (H-2^d) and C57BL/6 (H-2^b) mice, 6–8 weeks of age, were purchased from Experimental Animal Center of Jiangsu Province (Jiangsu, China). They were maintained with free access to pellet food and water in plastic cages at 21 ± 2 °C and kept on a 12-h light/dark cycle. Animal welfare and experimental procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (Ministry of Science and Technology of China, 2006) and the related ethical regulations of our university. Obaculactone (98%) and CsA were purchased from Sigma (St. Louis, MO) and the stock solution was prepared with dimethyl sulfoxide. Anti-mouse antibodies against CD3, CD25 and CD28 were from BD pharmingen (Becton Dickinson, San Diego, CA), and antibodies against CD4, Foxp3 and IFN-γ from eBioscience (San Diego, CA). IL-12 was from PeproTech (PeproTech Inc., Rocky Hill, NJ). All other chemicals were obtained from Sigma.

2.2. HPLC analysis and structural elucidation

HPLC analysis was applied on a Waters 600 series HPLC system consisting of a Waters 600 pump, a 2487 UV detector, an online degasser and an LC Work Station equipped with Empower TM software. Obaculactone was applied to YMC-pack Pro C18 column (5 μm, 150 mm × 4.6 mm, YMC Co., Ltd., Japan) and eluted with methanol–water (47:53, v/v). The effluents were detected at 204 nm. Column temperature was set up at 25° and the flow rate was 1 mL/min. The mobile phase was degassed by ultrasonic and filtered though a 0.22 μm membrane filter (Advantec, Tokyo Roshi Kaisha, Ltd., Japan). Before sample analysis, the column was stabilized with mobile phase for at least 30 min.

2.3. Skin transplantation and treatment

Full-thickness dorsal skin (1 cm^2) from C57BL/6 or BALB/c donor mice was transplanted on the dorsal flank area of BALB/c recipient mice and secured with a plastic adhesive bandage for 7 days [11]. Graft survival was assessed by daily visual inspection in a masked fashion. Rejection was defined as >70% necrosis of the transplanted skin surface, which gives the appearance with desiccation and shrinkage. The survival curve was monitored until the mice in all groups rejected grafts. Two treatment protocols were performed. In one protocol, different dosages of obaculactone or CsA (10 mg/kg) were intraperitoneally administered for 4 days (day −4, −3, −2, −1) before skin transplantation (day 0). In another protocol, these agents were given for 4 days (day 3, 4, 5, 6) after transplantation.

2.4. Histology

Skin grafts were harvested on day 7 after transplantation. Skin tissues were embedded in paraffin, and cut into 5 μm of sections and stained with hematoxylin and eosin.

2.5. Cytokine analysis by ELISA and CBA assay

For cytokine analysis, splenocytes harvested at day 20 after transplantation from recipients were restimulated by mitomycin C (Sigma) – treated donor spleen cells. The cell-free supernatants of individual wells were removed after 72 h. IFN-γ and IL-10 were measured by a specific ELISA kit from eBioscience according to the manufacture's instruction. Cytokine levels of IL-2, IL-17, TNFα, IL-6 and IL-4 were determined using Cytometric Bead Array (CBA) cytokine assay kit according to the manufacture as recommended by BD pharmingen.

2.6. Intracellular staining

For the intracellular IFN-γ staining, freshly isolated spleen cells were activated with phorbol 12-myristate 13-acetate (PMA; 50 ng/ml) and ionomycin (0.5 μg/ml) for 4 h in the presence of 1 μg/ml monensin (eBioscience). Cultured cells were stained with anti-CD3-PerCP. After permeabilization cells were stained by phycoerythrin (PE) conjugated anti-IFN-γ mAb or isotype control. Samples were analyzed by flow cytometry on a FACScan (Becton Dickinson).

Regulatory T cells were detected by Mouse Regulatory T cell Flow Cytometry staining Kit (ebioscience). According to manufacture's protocol, freshly isolated spleen cells were stained with PerCP-labelled anti-CD4 mAb and fluorescein isothiocyanate-labelled anti-CD25 mAb. After washing, these cells were then fixed and stained subsequently with PE-labelled anti Foxp3 mAb or PE-labelled rat IgG 2a mAb as nonspecific isotype control.

2.7. Regulatory T cell isolation and suppression assays [12]

A single-cell suspension was obtained by passing spleens cells through 38 μM cell strainers, and erythrocytes were lysed by Tris–NH_4Cl buffer. Regulatory T cells were isolated by $CD4^+CD25^+$ Regulatory T Cell Isolation Kit (Milteny, Germany). According to manufacture's protocol, $CD4^+$ T cells were pre-enriched by depletion of non-$CD4^+$ T cells in a cocktail method. For selecting $CD25^+$ and $CD25^-$ subsets, $CD4^+$ cells were labeled with CD25-PE, followed by anti-PE microbeads, and then loaded onto a MACS column. Positively selected $CD4^+CD25^+$ cells were >95% pure on flow cytometric analysis.

To determine the inhibitory activity, purified $CD4^+CD25^+$ or control cells were added, at indicated ratio, into the reporter $CD4^+CD25^-$ T cells (2×10^5/well), which were stimulated by anti-CD3 mAb (1 μg/ml) and anti-CD28 mAb (1 μg/ml) in triplicate in 96-well plates, and maintained in RPMI-1640 complete medium for 3 days in 5% CO_2 in air. [^{3}H] TdR (1 μCi/well) was added for the final 6 h, and incorporation of [^{3}H] TdR was assessed by liquid scintillation counting.

2.8. Western blotting

In brief, cells were washed with phosphate-buffered saline and lysed in the lysis buffer containing Triton X-100. After 10,000 × g centrifugation for 10 min, the protein content of the supernatant was determined by a BCA™ protein assay Kit (Pierce, Rochford, IL). The protein lysates were separated by 10% SDS-PAGE and subsequently electrotransferred onto a polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA). The membrane was blocked with 5% nonfat milk for 1–2 h at room temperature. The blocked membrane was incubated with the indicated primary antibodies. Primary antibodies used were against T-bet (1:1000 dilution, Santa Cruz Biotechnologies, Santa Cruz, CA) and GAPDH (1:1000 dilution, Santa Cruz Biotechnologies), and the secondary antibody were horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit immunoglobulin (1:10,000, KPL, Gaithersburg, MD). Protein bands were visualized using Western blotting detection system according to the manufacturer's instructions (Cell Signaling Technology, Beverly, MA).

2.9. Quantitative RT-PCR

RNA was extracted from cells using Trizol Reagent (Invitrogen, Carlsbad, CA). One microgram of RNA was reversely transcribed to cDNA. The primer sequences used in PCR were as follows: GAPDH, 5′-AACGACCCCTTCATTGAC and 3′-CACGACTCATACAGCACCT, T-bet, 5′-CTCAGGTGGCTGGCTTTC and 3′-ATTCGTTCCTGCCGCTTA, The PCR cycle conditions were: 94 °C for 30 s, 58 °C for 30 s, and

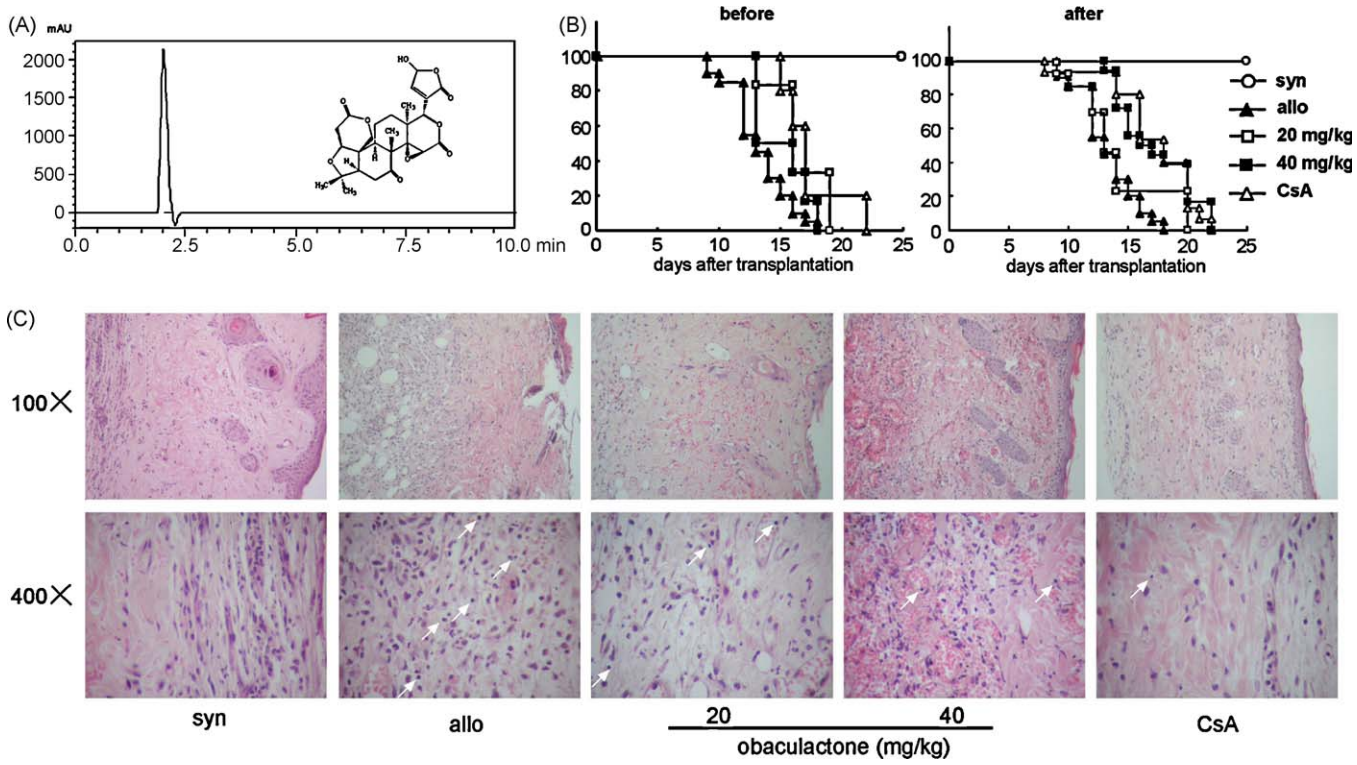

Fig. 1. Obaculactone treatment after transplantation prolonged skin graft survival in recipients. (A) The chemical structure of obaculactone and HPLC analysis of obaculactone. The purity of Obacualctone was confirmed to be 98%. (B) Survival of skin allografts. Different dosages of obaculactone were administered before or after skin transplantation for 4 days as described in Section 2, and then skin survival was monitored daily until graft shedding. (C) Pathology of skin allografts. Different dosages of obaculactone were administered after skin transplantation. At day 7, skin grafts were removed and then stained with H&E. Infiltrated cells were indicated by white arrows. Original magnification 100× and 400×.

72 °C for 30 s for 28 cycles. After the amplification, PCR products were separated by electrophoresis on 1.5% agarose gels and visualized using ethidium bromide. For quantitative real-time PCR analysis, amplification was carried out for 40 cycles of the same PCR conditions mentioned above and product was detected using SYBR Green I dye (Molecular Probes Inc., Eugene, OR). Reactions were run in triplicate using GAPDH as the internal RNA control on an ABI 7000 Thermocycler (Applied Biosystems Inc., Foster City, CA).

2.10. Statistical analysis

For the graft survival, Kaplan–Meier graph was constructed and log-rank comparison of the groups was used to calculate *P*-values. For other experiments, Data are expressed as means ± SD and statistical analyses were performed using one-way analysis of variance (ANOVA), followed by Student's two-tailed *t*-test. $P < 0.05$ was considered significant.

3. Results

3.1. Identification of obaculactone

Obaculactone bought from Sigma was subjected to HPLC analysis and structure determination. The purity of obaculactone was confirmed to be 98% by HPLC (Fig. 1A).

3.2. Effects of obaculactone treatment on skin allograft survival

A mouse skin graft model was used to evaluate the effects of obaculactone on allograft rejection. BALB/c mice were chosen as recipients and C57BL/6 mice as donors. Syngeneic skin grafts were accepted for at least 22 days of observation, and all allografts in the untreated control group were rejected with a median survival time (MST) of 13 days (Table 1). When administered daily before transplantation (day −4, −3, −2, −1), obaculactone failed to significantly prolong the survival of skin grafts (Fig. 1B). However, obaculactone caused a significant prolongation at the dose of 40 mg/kg with MST of 19 days when administered daily after transplantation (day 3, 4, 5, 6). In contrast, treatment with CsA (10 mg/kg) either before or after transplantation significantly prolonged graft survival with MST of 17 and 16 days, respectively. Further, skin grafts were harvested from the recipients 7 days after transplantation and analyzed for histological signs characterized by lymphocyte infiltration in the dermis, which was predominant in the grafts from the untreated mice (Fig. 1C). The infiltration was markedly attenuated in the grafts with treatment of 40 mg/kg of obaculactone after transplantation, whereas those grafts with treatment of 20 mg/kg of obaculactone demonstrated an intermediate infiltration.

Table 1
Effect of Obaculactone-treated before and after transplantation on allograft survival.

Group	Before transplantation		After transplantation	
	n	MST, *d*	*n*	MST, *d*
Syngeneic	10	>22	10	>22
Allogeneic	16	13	16	13
Obaculactone 20 mg kg^{-1}	6	16	13	14
Obaculactone 40 mg kg^{-1}	6	15	18	19**
CsA 10 mg kg^{-1}	6	17*	15	16*

* $p < 0.01$ vs allogeneic control (log-rank test).
** $p < 0.005$ vs allogeneic control (log-rank test).

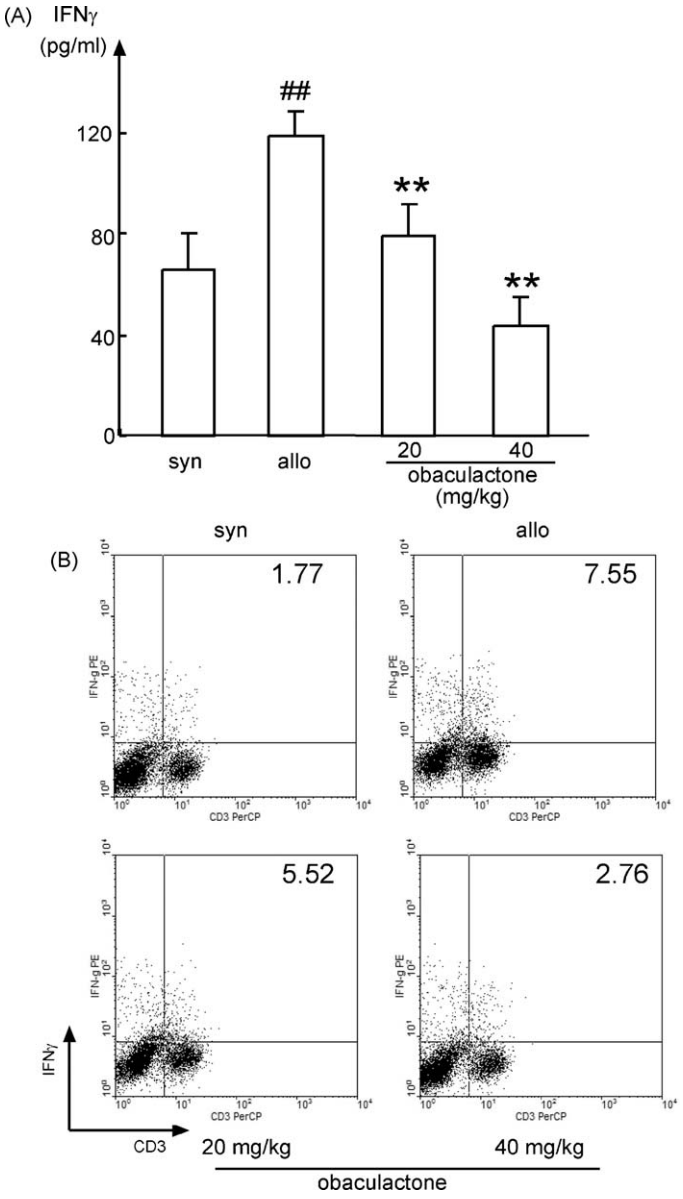

Fig. 2. Obaculactone treatment after transplantation inhibited IFN-γ production. Spleens were isolated from the mice receiving skin allograft at day 20 after transplantation. (A) IFN-γ production. The spleen cells of each group were restimulated with stimulator cells from C57BL/6 mice for 72 h. The supernatant was collected and IFN-γ was measured by ELISA. Data are shown as means ± SD of three independent experiments. $^{**}P < 0.01$ vs the allogeneic control, $^{\#\#}P < 0.01$ allogeneic vs syngeneic control. (B) IFN-γ-producing $CD3^+$ splenocytes. The spleen cells of each group were activated with PMA and Ionomycin in the presence of monensin. The intracellular IFN-γ was measured by FACS. One representative of two independent experiments was shown.

3.3. Inhibitory effects of obaculactone treatment after transplantation on Th1 and proinflammatory cytokine production

It has been postulated that Th1 cells are critically involved in the development of allograft rejection, whereas Th2 cells are involved in promoting graft survival [13,14]. Therefore, we first examined alloantigen-specific production of Th1 cytokine IFN-γ in the recipients of skin allografts at day 20 after transplantation. As shown in Fig. 2A, the production of IFN-γ was significantly increased in allograft group compared with syngraft group. Against the increase, obaculactone remarkably inhibited IFN-γ production in a dose-dependent manner (Fig. 2A). Intracellular staining and FACS analysis also showed the decrease in the percentage of IFN-γ-producing $CD3^+$ cells in splenocytes from obaculactone-treated mice (Fig. 2B). In addition, the other cytokines associated with allorejection, including IL-17, were examined. Splenocytes from 40 mg/kg of obaculactone-treated recipients compared with those from untreated recipients produced significantly lower amounts of proinflammatory cytokines IL-2, TNFα and IL-6 (Fig. 3). Notably, IL-17 and Th2 cytokine IL-4 were not markedly altered.

3.4. Up-regulatory effects of obaculactone treatment after transplantation on IL-10 production and $CD4^+CD25^+Foxp3^+$ Treg cell

Next, we examined alloantigen-specific production of regulatory cytokine IL-10 in the recipients of skin allografts. IL-10 production was markedly up-regulated by obaculactone at the dose of 40 mg/kg (Fig. 4A). The increased expression of IL-10 prompted us to investigate the effect of obaculactone on the $CD4^+CD25^+Foxp3^+$ Treg cell population. As shown in Fig. 4B, obaculactone significantly enhanced the ratios of $CD4^+CD25^+Foxp3^+$ Treg cells to $CD4^+$ T cells in the spleen in a dose-dependent manner (Fig. 4B), although no significant difference was observed in the absolute number of Treg cell

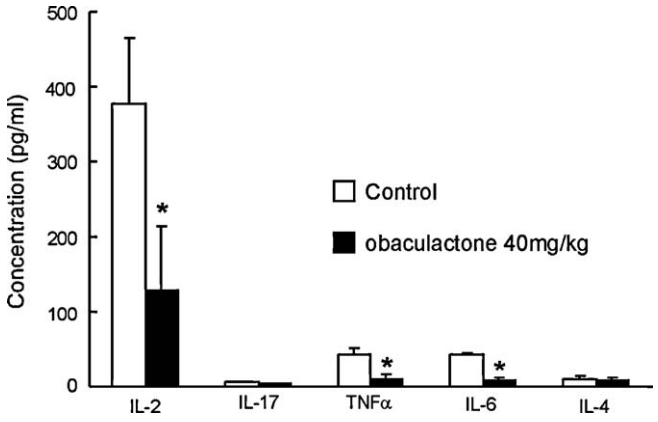

Fig. 3. Obaculactone treatment after transplantation down-regulated proinflammatory cytokine production. Spleen cells were freshly isolated from 40 mg/kg of obaculactone-treated mice receiving skin allograft at day 20 after transplantation, and then restimulated with stimulator cells for 72 h. Cytokine levels in the culture supernatant were measured by CBA assay. Data are shown as means ± SD of three independent experiments. $^{*}P < 0.05$ vs the allogeneic control.

among the groups (Fig. 4C). Since $CD4^{+}CD25^{+}$ Treg cells are capable of suppressing the proliferation of $CD4^{+}CD25^{-}$ T cells induced by antigens *in vitro*, splenic Treg cells were sorted by beads from obaculactone-treated and control recipients and their immunosuppressive ability was compared. As shown in Fig. 4D, regardless of $CD4^{+}CD25^{+}$ Treg cells from obaculactone-treated or control recipients, they showed comparably inhibitory function on the proliferation of $CD4^{+}CD25^{-}$ T cells stimulated by anti-CD3 plus anti-CD28.

3.5. Down-regulatory effect of obaculactone on T-bet expression in Th1 effector cells

T-bet is a crucial regulator of type 1-like immunity and plays an important role in the maintenance of Th1 effector function [15]. To investigate the potential mechanism of obaculactone as to how it improved skin allograft rejection by treatment after, but not before transplantation, the effect of obaculactone on T-bet expression in Th1 effector cells was examined. Because the generation of functional effector T cells in mixed lymphocyte reaction (MLR) takes at least 3 days [16], we firstly determined whether obaculactone could directly inhibit T-bet expression during the

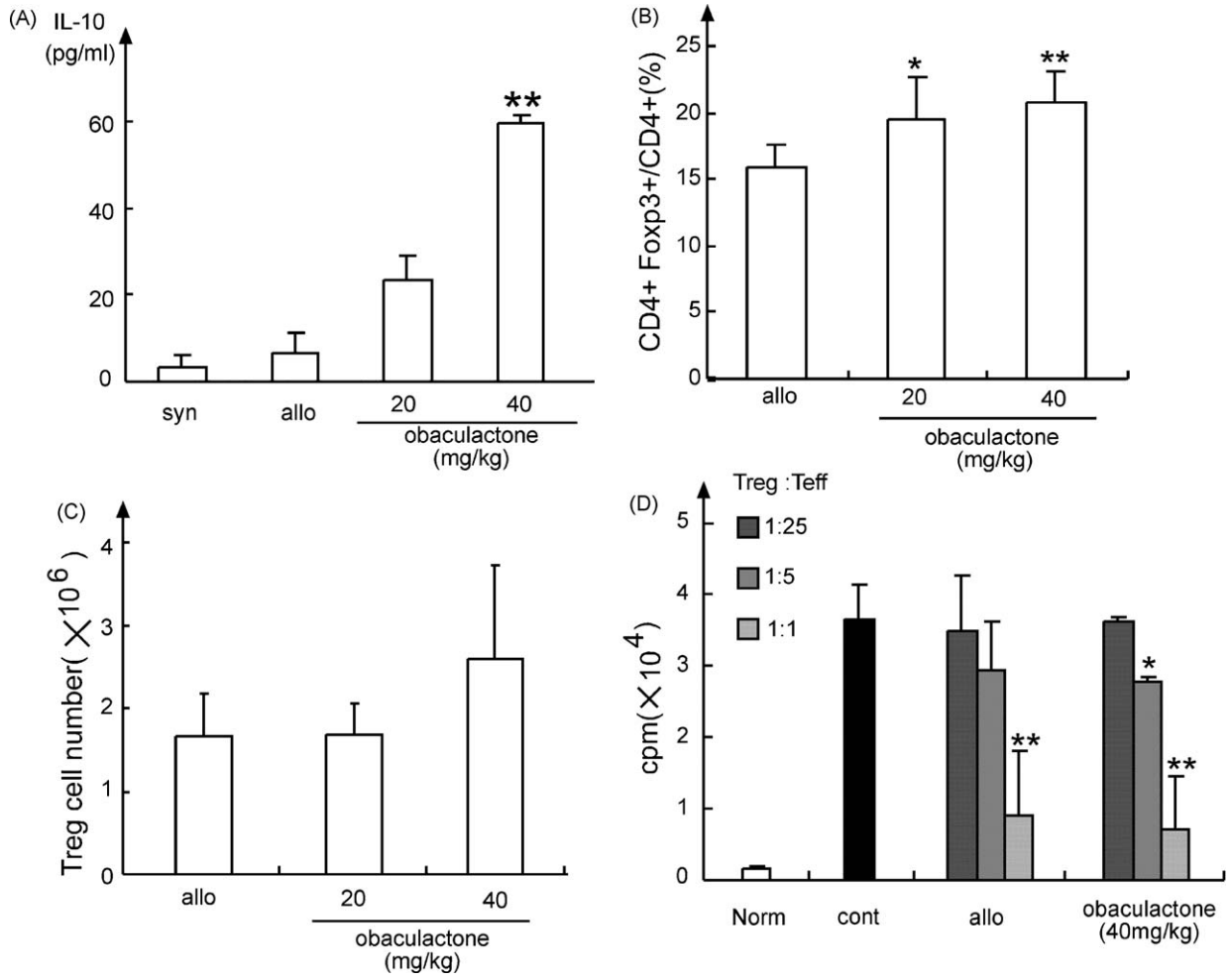

Fig. 4. Obaculactone treatment after transplantation increased IL-10 production and enhanced the percentage of $CD4^{+}CD25^{+}Foxp3^{+}$ Treg cells in the $CD4^{+}$ splenocytes. Spleens were isolated from the mice receiving skin allograft at day 20 after transplantation. (A) IL-10 production. The spleen cells of each group were restimulated with stimulator cells for 72 h. The supernatant was collected and IL-10 was measured by ELISA. Data are shown as means ± SD of three independent experiments. $^{**}P < 0.01$ vs the allogeneic control. (B) The proportion of $CD4^{+}Foxp3^{+}$ cells in $CD4^{+}$ spleen cells. $CD25^{+}CD4^{+}Foxp3^{+}$ regulatory T cells in the spleen were detected by mouse regulatory T cell flow cytometry staining kit. (C) The absolute number of $CD4^{+}Foxp3^{+}$ cells in the spleen. The absolute number was calculated according to flow cytometry. (D) Inhibitory function of Treg cells. Regulatory T cells were separated from the spleens. $CD4^{+}CD25^{-}$ T cells (Teff) were stimulated with anti-CD3 plus anti-CD28 antibodies in the presence of different proportion of $CD4^{+}CD25^{+}$ T cells (Treg). After 3 days, the proliferation was assessed by incorporation of $[^{3}H]$ thymidine. Data are shown as means ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ vs the control.

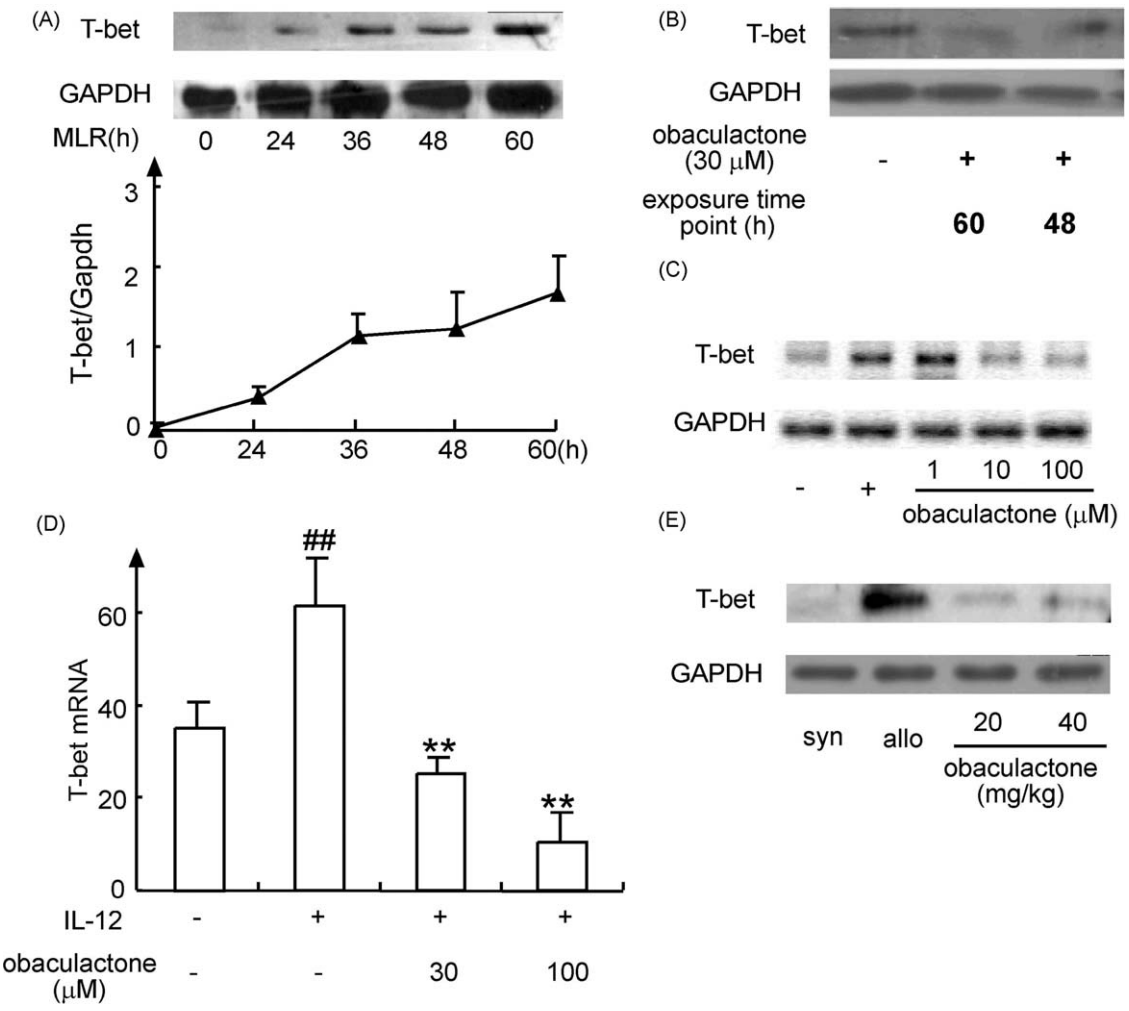

Fig. 5. Obaculactone down-regulated T-bet in effector Th1 cells. (A) T-bet kinetic expression during MLR. MLR was performed using spleen cells from Balb/c mice as responder cells and mitomycin C-pretreated spleen cells from C57BL/6 as stimulator cells. Cells were collected at indicated time. Protein was isolated and detected for T-bet by Western blotting. The polygraph below was the statistic result of three independent experiments. (B) Suppression of T-bet by Obacualctone added 48 h or 60 h after the beginning of MLR. Protein was collected at 72 h and T-bet was measured. (C) Splenocytes were isolated from the mice rejecting mismatched skin graft and restimulated by the splenocytes from syngenic mice (−) or from donor mice (+) in the absence or presence of Obacualctone. RNA was collected at 72 h and T-bet was measured by RT-PCR. (D) Lymph nodes were isolated from naïve Balb/c mice and primed with anti-CD3 plus anti-CD28 antibodies in the presence of IL-12 with Obacualctone added at 48 h. RNA was collected at 72 h. T-bet mRNA levels was measured by quantitative RT-PCR and normalized to gapdh mRNA levels. (E) The periphery lymph node cells were isolated 7 days after transplantation and the protein level of T-bet was measured by Western blotting. For all the above results, one representative of at least two experiments was shown.

late stage of MLR. Naïve Balb/c splenocytes were stimulated by mytomycin C-treated C57BL/6 spleocytes. As expected, T-bet protein was barely detectable at the beginning, but increased from 24 h to 60 h (Fig. 5A). Obaculactone nearly completely inhibited T-bet expression when added in MLR at 60 h (Fig. 5B). Further, spleen cells derived from the mice rejecting mismatched skin graft were used as primed T cells. When restimulated by donor mouse spleen cells, T-bet mRNA expression was suppressed by obaculactone in a dose-dependent manner (Fig. 5C). To confirm the inhibitory action of obaculactone on T-bet expression in Th1 effector cells, lymph node cells from naïve Balb/c mice were primed by anti-CD3 and anti-CD 28 antibodies in the presence of IL-12. Obaculactone was added at 48 h. As shown in Fig. 5D, T-bet mRNA level was reduced in a dose-dependent manner.

To verify that T-bet was suppressed *in vivo*, the lymph nodes were harvested from the recipients 7 days after transplantation and T-bet expression was examined by immunoblotting. T-bet protein level was markedly decreased in the obaculactone-treated recipients compared with an increase in the case of untreated recipients (Fig. 5E).

4. Discussion

Although graft survival has been greatly improved by the introduction of efficient immunosuppressive drugs in the last two decades, the side effects of long-term immunosuppression remain major obstacles for successful transplantation. There has been increasing interest to explore phytochemicals with therapeutic potential in alloimmune diseases in that they can be purified, synthesized and modified in chemical structure for new drug design, and often have low toxicity. It has been recently reported that Oleanolic Acid is a dietary nontoxic plant triterpenoid, which suppresses the production of proinflammatory cytokines and delays graft-specific immune responses to prolong islet allograft survival [17]. In this study, we demonstrated that obaculactone, a bitter compound accumulated in citrus seeds, has unique immunomodulatory properties as well as therapeutic potential for skin allograft rejection. Administering obaculactone after transplantation prolonged the survival of allografts, attenuated the infiltration of lymphocytes in the skin grafts, and reduced alloantigen-specific production of IFN-γ, IL-2, IL-6 and TNFα. But

obaculactone failed to protect the allografts when treated before transplantation. In contrast, CsA significantly prolonged graft survival in either treatment protocol. It should be noted that obaculactone is equally effective in contact hypersensitivity only when administered at the effector phase but not at the induction phase (our unpublished observation). These results suggest that obaculactone exhibits the immunosuppressive activity in a way different from CsA.

T-bet is a Th1-specific T-box transcription factor that not only induces Th1 development but also maintains Th1 effector function [15]. Numerous studies indicate that strongly increased T-bet expression is detected in patients with acute graft-vs-host disease or chronic allograft injury, suggesting the involvement of T-bet gene in transplant rejection [12,18]. In the current study, both *in vitro* and *in vivo* tests showed that obaculactone suppressed T-bet expression in Th1 effector cells. Obaculactone neither inhibited the lymphoproliferative response to mitogen concanavalin A nor altered the expression of activation markers including CD25 and CD69, but reduced IFN-γ production by spleen cells (data not shown). During the maturation of Th1 effector cell in MLR, T-bet protein expression continued to increase and was nearly completely inhibited if obaculactone was added at 60 h. In fact, we found that IFN-γ production was also efficiently inhibited when obaculactone was added at the late stage of MLR (Supplementary Data 1). These results suggest that obaculactone could suppress IFN-γ production in T cells through a pathway that is sensitive to T-bet levels. Obaculactone could induce T-bet degradation (Supplementary Data 2). Recent studies show that T-bet negatively regulates the development of IL-17-producing Th17 cells and its deficiency accelerates cardiac allograft rejection in a mouse model of chronic allograft vasculopathy. However, no significant deference was observed in IL-17 production in the spleen between obaculactone-treated and untreated recipients. It is possibly due to the difference in partial verse complete T-bet deficiency and timing of inhibitory action. In other study, silencing T-bet by small interfering RNA after disease onset improved experimental autoimmune encephalomyelitis and inhibited Th17 cells via regulation of IL-23R [19].

With divergent effector functions, Th1 and Th2 cells are mutually exclusive. Not surprisingly, a Th2-predominant environment seems to be established by obaculactone treatment after transplantation because alloantigen-specific production of IL-10 was markedly up-regulated whereas IFN-γ was reduced. IL-10 has been considered as a negative regulator of immune response and produced by a variety of cell types, including $CD4^+CD25^+Foxp3^+$ Treg cells [20,21]. We found that obaculactone treatment elevated the percentage of Treg in splenic $CD4^+$ T cells while keeping these cells function. Moreover, the absolute cell number of Treg exhibited an increasing tendency in the spleens from the recipients treated by 40 mg/kg of obaculactone. Numerous studies indicate that CsA and tacrolimus (FK506) can significantly decrease the cell numbers and function of $CD4^+CD25^+$ Treg cells *in vivo* and fail to induce transplantation tolerance [22,23]. Such suppression of Treg is related to the blockade of IL-2 pathway because these calcineurin inhibitors inhibit T cell activation through the IL-2 deprivation, and IL-2 is an important cytokine for the survival and proliferation of Tregs [24]. Unlike CsA, obaculactone has no effect on T cell activation despite its immunosuppressive activity. Enhancement of IL-10 and Treg induced by obaculactone is possibly responsible for the delayed skin allorejection and the potential to promote transplantation tolerance.

Nonspecific inhibition on T cells prolongs the survival time of the graft, but interferes with normal immune response. Many selective strategies for transplantation have been reported feasible and promising, for example, selective activated alloreactive T-cell depletion by using anti-CD25 or CD69 antibodies [25,26]. Obaculactone, which has unique immunomodulatory properties, might be useful as an efficacious natural compound for transplantation and as a tool for investigating selective immune response.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2010.03.028.

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