



Icaritin inhibits T cell activation and prolongs skin allograft survival in mice

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ABSTRACT

Icaritin is a native compound from *Epimedium Genus*, a traditional Chinese herbal medicine which is effective in treating asthma, autoimmune diseases and viral infections. In the present paper, the immunosuppressive effects of icaritin were found through *in vitro* and *in vivo* studies. Icaritin could dose-dependently inhibit murine CD4⁺ T cells proliferation stimulated with mitogens or specific antigen ovabumin (OVA). Icaritin at 0.25–25 μ M could down-regulate T cell activation marker CD25 expression and inhibit IL-2 production. It could also reduce the Th1 cytokine IFN- γ production significantly if the T cells were activated by ConA or anti-CD3; while the inhibition of IL-4 secretion was only seen on anti-CD3 activated T cells treated with low concentrations of icaritin. *In vivo* study showed that treatment of icaritin at 10 mg/kg/day on mice could suppress the immune response with prolonged allograft skin survival. Further study demonstrated that it reduced the alloantigen-induced splenocytes proliferation and Th1/Th2 cytokines. It could also increase NF-AT luciferase activity in Jurkat-NF-AT-luc T cells. The above results suggested that icaritin might be used to treat Th1 dominated immune diseases by interfering T cells activation with mechanism different to CsA.

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1. Introduction

Icaritin (Ica, Fig. 1) is a hydrolytic product of icariin from *Epimedium Genus*, a traditional Chinese herbal medicine. *Epimedium Genus* has a broad range of therapeutic applications in genital, cardiovascular, endocrine, bronchial, urinary, and immune systems [1].

Icaritin has been shown to possess many pharmacological and biological activities. It could suppress the osteoclasts differentiation [2] and prevent steroid-associated osteonecrosis [3]; promote cardiac differentiation of mouse embryonic stem (ES) cells via reactive oxygen species (ROS) signaling [4]; stimulate neuronal differentiation [5–7]; inhibit human breast and prostate cells proliferation by sustaining ERK1/2 activation and inducing apoptosis [8–11]; and exhibit estrogen-like activity in an estrogen receptor positive breast cancer cell line MCF-7 [12,13]. However, to our best knowledge there is no report about the effect of icaritin on immune activation.

Dysregulated activation of T cells is the hallmark of many immune mediated disorders [14]. Full activation of T cells requires the presentation of an antigenic stimulus via antigen presenting cell (APC) to T cells and the co-stimulatory signals (CD28, CD80 and CD86) on APC [15]. Following the stimulation, the activation marker such as CD25 can be up-regulated. As the high affinity α chain of IL-2 receptor, the binding

of CD25 to the IL-2 can trigger a cascade response, resulting in T cell proliferation and additional IL-2 production [16]. IL-2 is also defined as a Th1 cytokine. There are elevated Th1 cytokines (IFN- γ , IL-2, etc.) and Th2 cytokines (IL-4, IL-10, etc.) secreted by activated T helper cells or a higher Th1/Th2 ratio in various of immune overwhelming diseases, such as autoimmune, allergy and transplantation rejection [17–19]. Therapeutic interventions to such diseases are often designed to reduce the threshold of T cell activation [16]. Therefore, immunosuppressive drugs, like cyclosporine A (CsA) and dexamethasone (Dex), are often used in these immune overwhelming diseases [20,21].

In the present study, we found that icaritin at a quite low concentration could act as an immunosuppressant to reduce T cell proliferation, IL-2/CD25 expression, and Th1 cytokines production *in vitro*. To confirm the immune suppressive effect of icaritin *in vivo*, mouse skin transplantation, the standard model to assay host T cell responses to MHC-disparate donor antigens [22], was selected. NF-AT-luc T cell line was used to study the mechanism of action of icaritin on T cell activation in contrast to CsA. Our results suggested that icaritin might be used as an immune suppressor for the treatment of immune overwhelming diseases with mechanism different to CsA.

2. Methods

2.1. Mice

Female BALB/c mice and C57BL/6 mice at the age of 7–8 weeks were purchased from the Animal Laboratory Center of Southern

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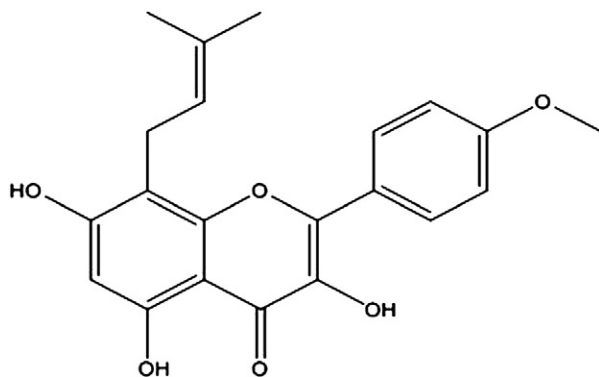


Fig. 1. The chemical structure of icaritin.

Medical University, China. The DO11.10 ovalbumin transgenic (OVA Tg) mice were obtained from Nanjing University, China. The protocol of mice experiments were approved by the Animal Ethics Committee of Southern Medical University, China.

2.2. Materials

Icaritin (Ica, HPLC purity >98%) was obtained from Shanghai Tauto Biotech (Shanghai, China). Anti-mouse CD3, anti-mouse CD4-PE and anti-mouse CD25-APC were purchased from eBiosciences (San Diego, CA). Concanavalin A (ConA), Dex, CsA and ovalbumin (OVA) were purchased from Sigma-Aldrich (St. Louis, MO). 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) was obtained from Invitrogen (Carlsbad, CA). RPMI 1640 medium, fetal bovine serum (FBS), penicillin and streptomycin were purchased from Gibco Lab (Rockville, MD). The IL-2, IL-4 and IFN- γ ELISA kits were purchased from eBiosciences.

2.3. Determination of murine splenocytes and CD4⁺CD25⁺ effector T cells proliferation by CCK-8 assay

The spleen was separated from female BALB/c mice and the red cells were removed by NH₄Cl. To test the effects of icaritin on the ConA (2 μ g/ml), anti-CD3 (0.1 μ g/ml) stimulated or not stimulated cells proliferation, splenocytes (3×10^5 /well) were plated into 96-well plate with or without stimulator and administrated Dex (1 μ M) or icaritin (0.25–25 μ M) simultaneously for 48 h. To measure the effects of icaritin on antigen specific splenocytes proliferation, 3×10^5 splenocytes of OVA-specific DO11.10 Tg mice [23] were incubated with icaritin at various concentrations in wells of 96-well plates at the present of chicken ovalbumin (OVA, 100 μ g/ml) at 37 °C for 72 h. The cell proliferation assays were performed using Cell Counting Kit-8 (CCK-8) kit obtained from Dojindo Inc (Kumamoto, Japan). At the indicated time points, the absorbance at 450 nm in triplicate wells were measured by an ELISA plate reader to indicate cell proliferation.

The CD4⁺CD25⁺ effector T cells were purified by Mouse CD4⁺CD25⁺Regulatory T cell Isolation Kit (Miltenyi Biotec, Germany) according to the manual instruction. The purity of CD4⁺CD25⁺ effector T cells analyzed by FACS were higher than 90%. CD4⁺CD25⁺ effector T cells (2.5×10^5 /well) were plated into 96-well plate with ConA (2 μ g/ml) and icaritin (0.025–2.5 μ M) simultaneously. The cell proliferation assays were performed by using CCK-8 kit after 72 h incubation.

2.4. Analysis of CD4⁺ T cells proliferation by CFSE-stain FACS

One ml splenocytes (2×10^7) were labeled with 1 μ M CFSE for 10 min away from light at 37 °C. The cells were then washed by RPMI 1640 complete medium and stimulated with ConA (2 μ g/ml). Icaritin was added at concentrations of 0.25, 2.5, 25 μ M with ConA

simultaneously. Three days later, the cells were collected and stained with anti-CD4-PE, measured by flow cytometry (FACS Calibur, Becton Dickinson). Cell division was presented by the dilution of CFSE. Proliferation rates (% of relative cells) were calculated in both CD4⁺ and CD4⁺ splenocytes.

2.5. Measurement of CD4⁺ T cells activation marker CD25

ConA (2.0 μ g/ml) or anti-CD3 (0.1 μ g/ml) was added with icaritin simultaneously to the splenocytes cultured in a 24-well culture plate. After incubation for 24 h, the cells were stained with anti-CD4-PE and anti-CD25-FITC in 100 μ L PBS/10⁶ cells away from light for 30 min. The percentage of CD25 molecules on the surface of CD4⁺ lymphocytes was evaluated by FACS analysis.

2.6. Detection of ConA or anti-CD3 induced IL-2, IFN- γ and IL-4 production by enzyme-linked immunosorbent assay (ELISA)

After cultured with simulators (ConA or anti-CD3) and tested compounds in 24-well plate at 37 °C for 48 h, the splenocytes were centrifuged and the supernatant was taken out for assay. Th1

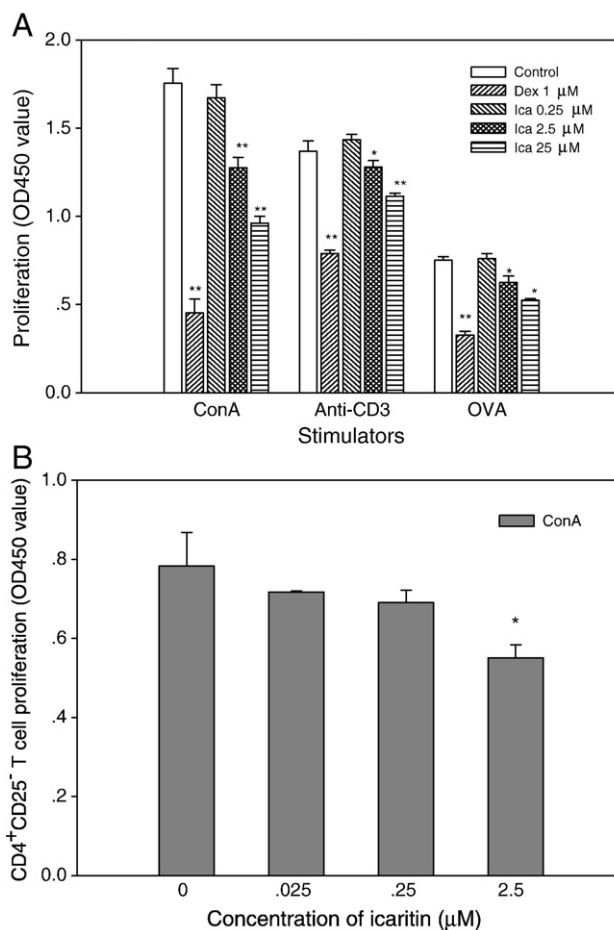


Fig. 2. Effects of icaritin on splenocytes and CD4⁺CD25⁺ effector T cells proliferation by CCK-8 assay. Splenocytes or purified CD4⁺CD25⁺ effector T cells were cultured with icaritin and different stimulators, including ConA, anti-CD3, OVA for 48 or 72 h as described in Methods. Four hours before the end of cell culture, CCK-8 was added and OD450 was measured to indicate the cell proliferation. (A) Splenocytes proliferations with different stimulators and drugs. The stimulation indexes (proliferation of cells with stimulator only/proliferation of cells with medium only) of ConA, anti-CD3 and OVA group are 4.07, 3.16 and 2.32 respectively. (B) CD4⁺CD25⁺ effector T cells proliferation stimulated with ConA. The stimulation index of ConA is 2.90. Data points present as mean $\pm$ SD from 3 replicates and 3 such experiments were carried out. *P < 0.05, as compared to relative stimulation group without any drugs; **P < 0.01, as compared to relative stimulated group without any drugs.

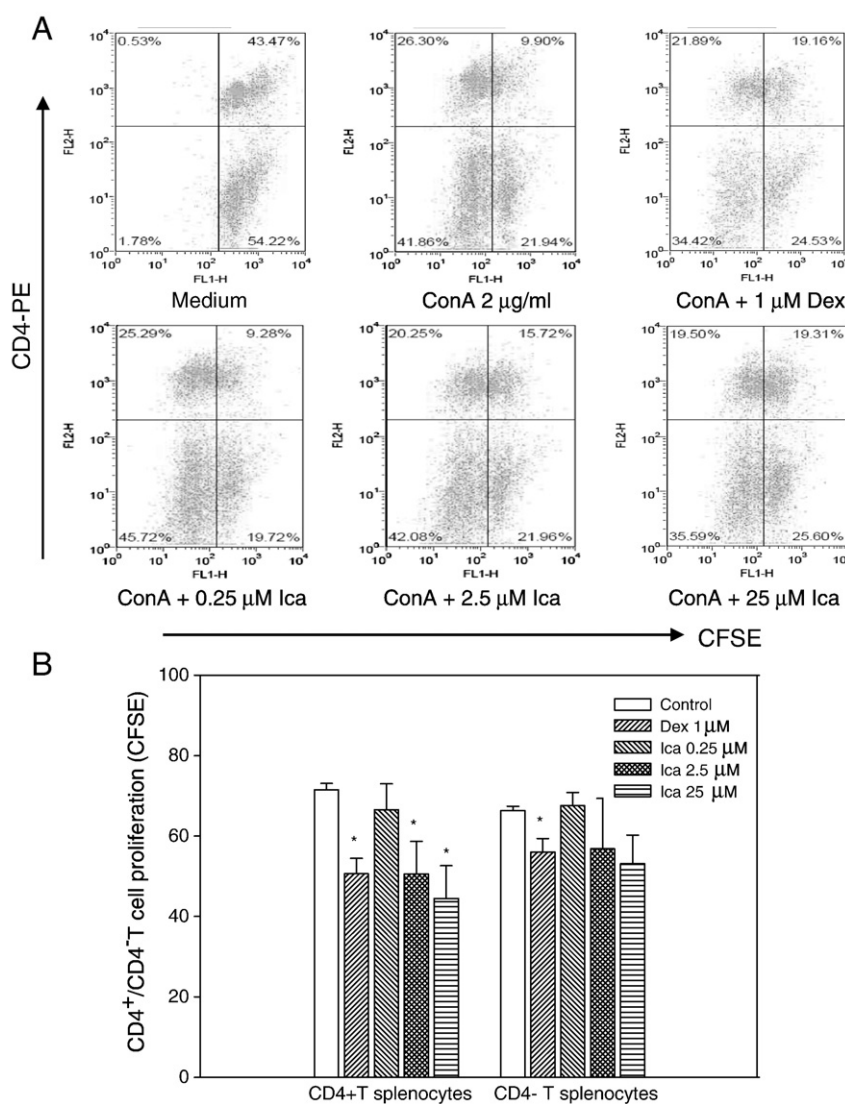


Fig. 3. Icaritin inhibited CD4⁺/CD4⁻ T cells proliferation by CFSE analysis. Splenocytes were labeled with 1 μ M CFSE, washed and stimulated with ConA (2 μ g/ml). Icaritin was added with ConA simultaneously. Three days later, the cells were collected and stained with anti-CD4-PE, measured by flow cytometry. (A) The dot plot presents CFSE labeled proliferation. The dilution of CFSE indicates the cell division. (B) Proliferation rates of CD4⁺ or CD4⁻ T cells, which were calculated by CellQuest software. * $P < 0.05$, as compared to ConA stimulated cells; ** $P < 0.01$, as compared to ConA stimulated cells.

cytokines IL-2 and IFN- γ , as well as Th2 cytokine IL-4, were measured by appropriate ELISA kits (eBioscience) according the manufacturer's instruction.

2.7. Murine allogeneic skin transplantation

The skin transplant model is a quick and easy method to monitor allogeneic T cell responses and to evaluate the efficiency of immunosuppressive agents. The skin transplantation was performed following the protocol reported previously [22]. Briefly, a piece of ear skin from C57BL/6 mice was grafted on to the back of the BALB/c mice. The signs of rejection were monitored daily from day 6 after transplantation. The tested compounds in saline were orally administrated 2 weeks before the transplantation and continued daily for 7 days after the engraftment.

2.8. Detection of alloantigen-induced splenocytes proliferation and Th1/Th2 cytokines production

Splenocytes of C57BL/6 mice were incubated with 50 μ g/ml mitomycin C (Sigma) in 37 $^{\circ}$ C water bath for 40 min [24]. Mitomycin

C treated C57BL/6 splenocytes could not proliferate after ConA stimulation, which were prepared as APC to present alloantigens to T cells of BALB/c mice in this experiment. BALB/c splenocytes at 3×10^5 /well were plated into 96-well plate with equal number of mitomycin C treated C57BL/6 splenocytes. Simultaneously, CsA (1 μ M), Dex (1 μ M) or icaritin (0.25–25 μ M) was added. Three days later, the plates were centrifuged at 1200 rpm for 5 min to collect the supernatant to measure IL-2, IFN- γ and IL-4 by ELISA kit. Cells were then added with 95 μ l fresh culture medium and 5 μ l CCK-8 detecting solution to incubate for further 8 h. The alloantigen-induced proliferation was indicated by OD450 value as described above.

2.9. NF-AT luciferase activity assay

Jurkat-NF-AT-luc cells (Kindly provided by Dr. Michel J. Tremblay, Laval University, Canada) at 1×10^5 /well were cultured in 96-well flat bottom plates, stimulated with 30 nM PDB and 1 μ M ionomycin, incubated with icaritin (0.25–25 μ M), 1 μ M CsA or 1 μ M Dex. After 8 h of stimulation, cells were washed with PBS and lysed with 20 μ l lysing reagent included in the luciferase kit (Promega, Madison, WI). Aliquots of cell lysates were transferred to 96-well flat bottom luminometer

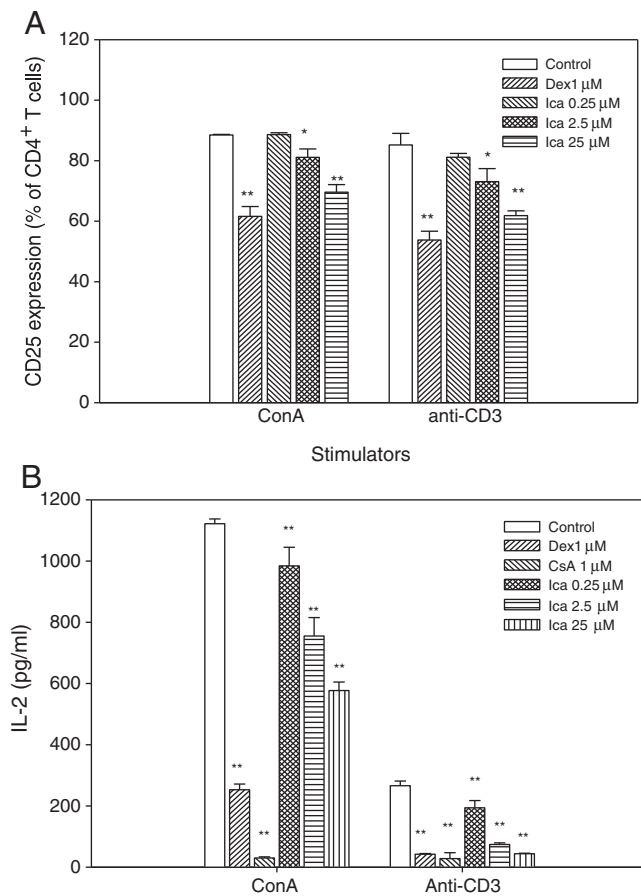


Fig. 4. Icaritin down-regulated ConA or anti-CD3 stimulated CD25 expression and IL-2 production. ConA (2.0 μ g/ml) or anti-CD3 (0.1 μ g/ml) was added with icaritin simultaneously to the splenocytes. After incubation for 24 h, the cells were stained with anti-CD4-PE and anti-CD25-APC. (A) The percentage of CD25 molecules on the surface of CD4⁺ lymphocytes was evaluated by FACS analysis. (B) The measurement of IL-2 production by ELISA after incubation for 48 h. * P <0.05, as compared to relative stimulation group without any drugs; ** P <0.01, as compared to relative stimulation group without any drugs.

plates (Costar), followed by the addition of luciferase substrate (Promega). The luciferase activity was measured in a microplate luminometer (Genios Pro, Tecan, US).

2.10. Statistical analysis

The results are shown as mean $\pm$ SD. Student's t test was used to compare differences between two groups *in vitro*. Kaplan–Meier survival analysis and log-rank statistical analysis in GraphPad Prism software were selected to draw graph and compare the differences between drug treated group and model group after transplantation *in vivo*. The p value <0.05 was considered significantly different.

3. Results

3.1. Icaritin inhibited splenocytes and CD4⁺ T cell proliferation

Icaritin could inhibit ConA (2 μ g/ml) or anti-CD3 (0.1 μ g/ml) stimulated mouse splenocytes proliferation by CCK-8 assay. The ovabumin (OVA) stimulated antigen specific splenocytes proliferation of DO11.10 mice could also reduced by icaritin (Fig. 2A). In addition, icaritin could down-regulate the proliferation of ConA activated CD4⁺CD25⁺ effector T cells (Fig. 2B). We then tested whether icaritin had direct effects on CD4⁺ T cell proliferation by CFSE analysis. Icaritin was found to inhibit ConA stimulated CD4⁺ T cell proliferation at concentration of 2.5 μ M and 25 μ M. As

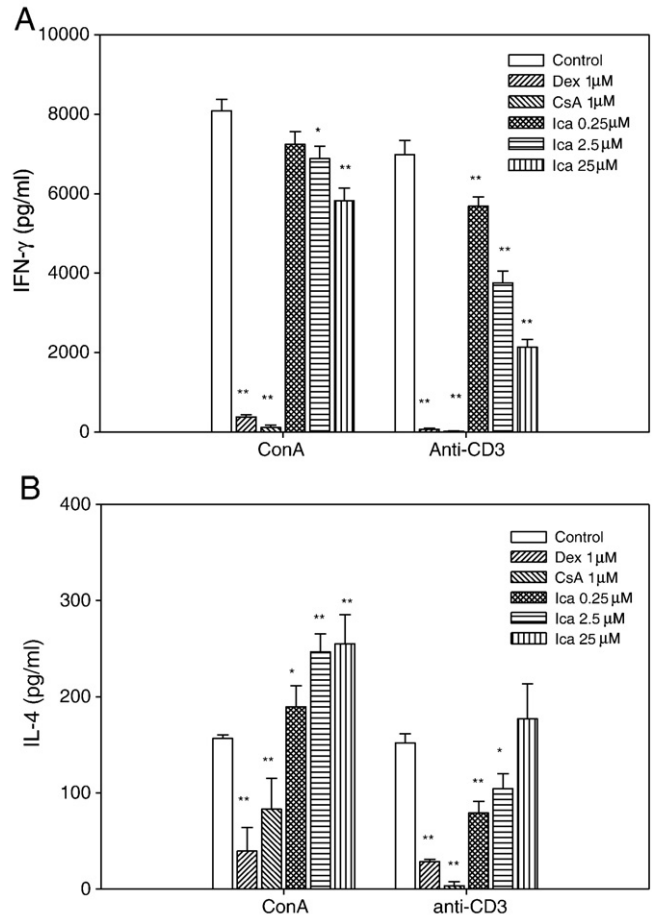


Fig. 5. Interfering of ConA or anti-CD3 stimulated Th1/Th2 cytokines by icaritin. The splenocytes were cultured with icaritin and stimulators such as ConA (2 μ g/ml) or anti-CD3 (0.1 μ g/ml) for 48 h, then the supernatant was taken out to detect Th1 cytokines IFN- γ (A), as well as Th2 cytokine IL-4 (B) by ELISA. * P <0.05, as compared to relative stimulation group without any drugs, ** P <0.01, as compared to relative stimulation group without any drugs.

shown in Fig. 3, a slightly stronger inhibitory effect on CD4⁺ T cells proliferation than CD4⁺ T cells proliferation could be observed. Therefore, our results showed that icaritin reduced antigen specific and non-antigen specific splenocytes proliferations obviously at concentration of 2.5 and 25 μ M, though it showed no effect at 0.25 μ M.

3.2. Icaritin down-regulated ConA or anti-CD3 induced IL-2 production and CD25 expression

The binding of IL-2 to CD25 triggers T cells proliferation. In the present study, the level of CD25 expressed on CD4⁺ T cells was decreased by icaritin from the concentration of 2.5 μ M (Fig. 4A). It also reduced the IL-2 production of CD4⁺ T cells significantly from the concentration of 0.25 μ M after activated with ConA or anti-CD3 (Fig. 4B). The inhibition rates of 0.25, 2.5, 25 μ M icaritin on IL-2 production were 13.3%, 22.7%, 48.6% after ConA stimulation and 27.1%, 72.2%, 83.5% after anti-CD3 stimulation respectively (Fig. 4B).

3.3. Icaritin interfered ConA or anti-CD3 induced Th1/Th2 cytokines production

IFN- γ and IL-4 belong to typical Th1 and Th2 cytokines, respectively. IFN- γ /IL-4 is a commonly used Th1/Th2 cytokine pair. We found a significant reduction of Th1 cytokine IFN- γ production in anti-CD3 stimulated splenocytes in response to icaritin from

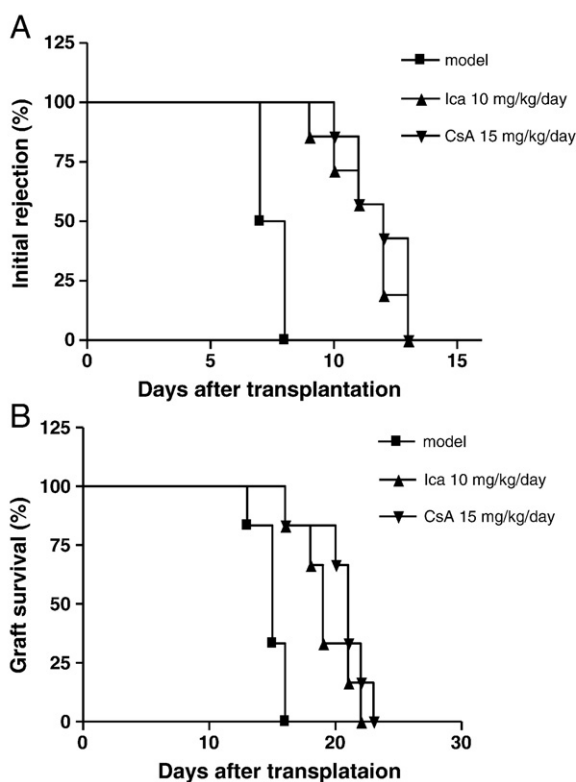


Fig. 6. Icaritin prolonged allograft skin survival. Ear skin from C57BL/6 mice was grafted on to the back of the BALB/c mice (6 mice per group). The signs of rejection were monitored daily from day 6 after transplantation. The drugs in saline were orally administered 2 weeks before transplantation and continued daily for 7 days after engraftment. (A) Percentage of graft initial rejection at different time after skin transplantation. (B) Percentage of graft survival at different time after skin transplantation.

0.25 μ M. Meanwhile, it was observed to show inhibition on IFN- γ production after ConA stimulation from the concentration of 2.5 μ M (Fig. 5A). On the other hand, icaritin inhibited Th2 cytokine IL-4 production at lower concentrations of 0.25 and 2.5 μ M after anti-CD3 stimulation, but it increased the IL-4 production after ConA stimulation (Fig. 5B).

3.4. Icaritin improved allograft skin survival

An ear skin transplantation model in mice was established to evaluate the *in vivo* immunosuppressive effects of icaritin. The time when the perimeter of the grafted skin became sclerotic was considered as initial rejection time. The days before the allograft skin completely shed from the recipient were considered as the graft survival time [25]. As shown in Fig. 6, icaritin at 10 mg/kg/day could significantly delay the initial rejection time ($P < 0.01$) and prolong the graft survival time ($P < 0.01$) when compared with control model group, which was close to the effect of CsA at 15 mg/kg/day. It was also observed that CsA treated mice became restless, fur-cocking and leptystic, but these signs were not occurred in the mice treated with icaritin.

3.5. Icaritin inhibited alloantigen-induced splenocytes proliferation and Th1/Th2 cytokines production

When cultured BALB/c splenocytes with mitomycin treated C57BL/6 splenocytes, we found that 2.5 and 25 μ M of icaritin could reduce the alloantigen-induced splenocytes proliferation (Fig. 7A). Icaritin could also dose-dependently inhibit alloantigen-induced Th1 cytokines such as IL-2 (Fig. 7B) and IFN- γ (Fig. 7C) secretion from 0.25 μ M. The inhibition rates of 0.25 μ M icaritin on IFN- γ and IL-2

production are 49.7% and 53.5%, respectively. Regarding to the Th2 cytokines, icaritin could inhibit alloantigen-induced IL-4 production from 0.25 μ M, though the IL-4 increases with increasing doses of icaritin in a trend opposite to the IFN- γ (Fig. 7D).

3.6. Icaritin increased NF-AT expression in T cells

NF-AT was identified as a target for the immunosuppressants CsA and FK506 [26]. NF-AT is also a key transcription factor in CD4⁺ T cell activation, Th1/Th2 differentiation and T cell tolerance induction [27,28]. NF-AT complex participated the production of several cytokines (including IL-2, IFN- γ , IL-4, etc.) by interacting with other transcript factors [27]. As a direct calcineurin inhibitor, CsA blocks calcineurin dependent dephosphorylation of cytoplasmic NF-AT and its nuclear translocation [29]. Here, we used a constructed NF-AT luciferase transcription system to see if icaritin has the same inhibitory mechanism with CsA. Interestingly, icaritin could significantly increase the NF-AT luciferase activity at high concentration of 25 μ M in PDB plus ionomycin stimulated Jurkat T cells. At the same time, CsA at 1 μ M completely blocked the increased NF-AT transcription, and Dex (1 μ M) showed no effects on NF-AT activity (Fig. 8).

4. Discussion

In the present study, we observed for the first time the immunosuppressive effect of icaritin *in vitro* which directly reduced T cell proliferation, IL-2/CD25 expression and Th1 cytokines production stimulated by mitogens or alloantigens. The protective effect on the allograft skin rejection also provided an evidence of its immunosuppression *in vivo*.

IL-2 was identified as a T cell growth factor since 1976. It is required for T cell proliferation and induction of effector functions [30]. CD25 is the alpha chain of IL-2 receptor, also constitutively expressed on CD4⁺CD25⁺Foxp3 regulatory T cells (Treg) [30]. Treg usually comprises only 5–10% of peripheral CD4⁺ T cells. Unlike CD4⁺CD25⁺ effector T cells, Treg cannot proliferate after stimulation though it becomes immune suppressive. As a T cell activation marker, CD25 could be dramatically up-regulated on effector T cell surface when stimulated with anti-CD3, ConA, PKC agonists and specific antigens. The increase of CD25 on CD4⁺ T cells could represent the CD4⁺CD25⁺ T cells activation in splenocytes culture. Therefore, the inhibition of icaritin on T cells proliferation might attribute to the suppression of IL-2 and CD25.

In addition to IL-2, icaritin also showed obvious suppressive effect on another Th1 cytokine IFN- γ . The inhibition on Th1 cytokines production by icaritin after anti-CD3 or alloantigen stimulation was much stronger than that after ConA stimulation. ConA can stimulate CD4⁺CD25⁺ effector T cell proliferation through TCR signal only, while fully activating T cells by anti-CD3 and alloantigens need co-stimulatory signals supported from APC in addition to the TCR signal [31]. Therefore, icaritin might suppress APC to facilitate the reduction of T cells activation. Macrophage is one of the most important APC. We also observed that icaritin could decrease NF- κ B activity in Raw264.7 murine macrophages after LPS stimulation (data not shown), which demonstrated the effects of icaritin might be due to the inhibition of the activation of APC.

Th1/Th2 cytokines play important roles in autoimmunity, allergy and organ transplantation. Th1 cells contribute to the pathogenesis of several organ-specific autoimmune diseases, whereas Th2 cells may inhibit disease progression [32]. Th1 cytokines can stimulate delayed-type hypersensitivity (DTH) skin reactions [33]. Acute allograft rejection is usually associated with the Th1 like cytokines, such as IL-2 and IFN- γ , although Th2 cytokines IL-4 can also be elevated in rejecting grafts. Treatment with immunosuppressants, such as tacrolimus (FK-506) and sirolimus (rapamycin), could decrease Th1 cytokines production and prolong allograft survival [26]. Cyclosporin

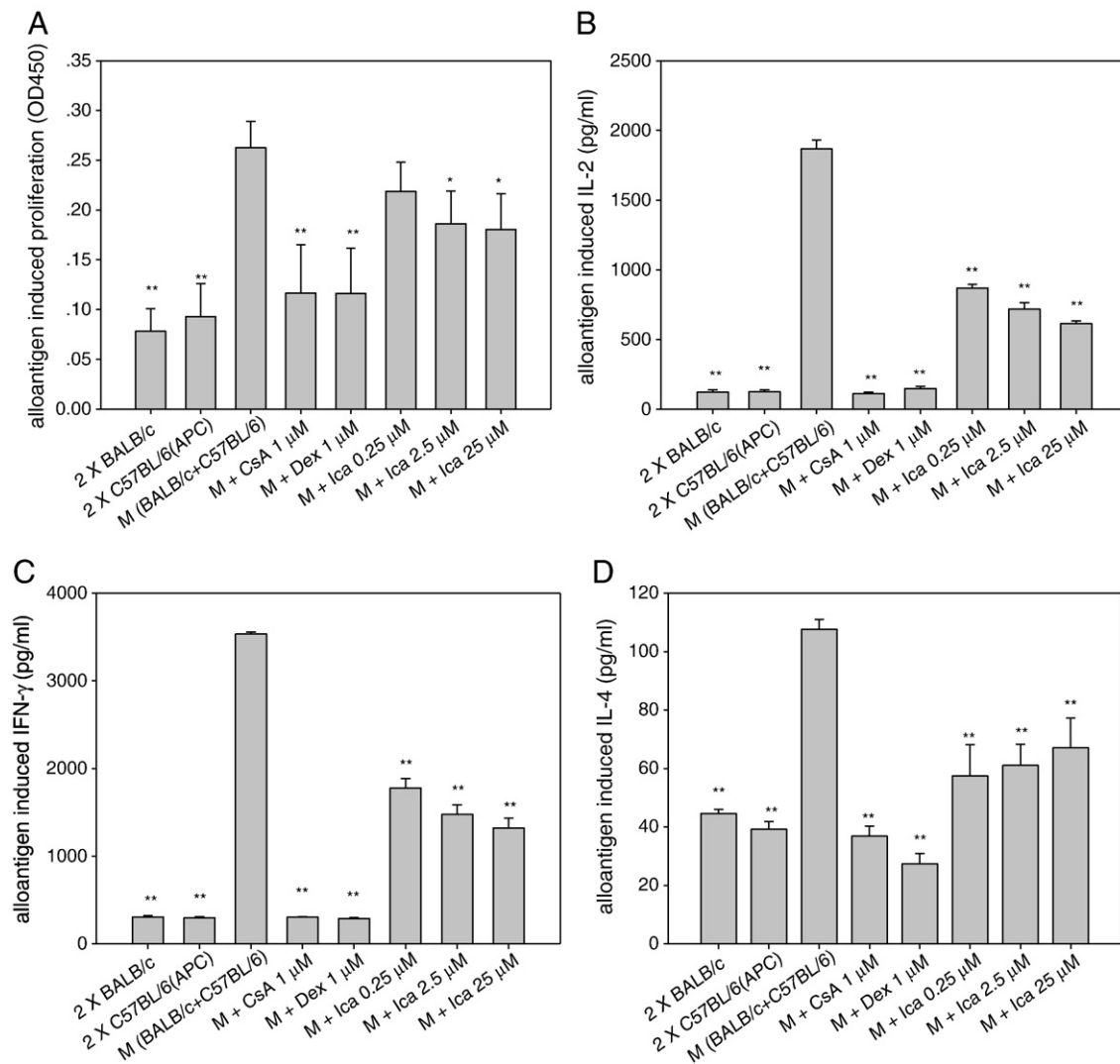


Fig. 7. Icaritin inhibited alloantigen-induced proliferation and Th1/Th2 cytokines production. BALB/c mice splenocytes (3×10^5 /well) were cultured with equal numbers of mitomycin C treated C57BL/6 mice splenocytes (APC) to provide alloantigens. Three days later, alloantigen-induced cell proliferation was detected by CCK-8 assay (A), the supernatants were collected to measure IL-2 (B), IFN-γ (C), IL-4 (D) by ELISA. 2×BALB/c or 2×C57BL/6 (APC) indicated double numbers of splenocytes (6×10^5 /well) of BALB/c or mitomycin C treated C57BL/6 mice to each well. * $P < 0.05$, as compared to M group (Mixed culture of BALB/C and mitomycin C treated C57BL/6 splenocytes), ** $P < 0.01$, as compared to the M group.

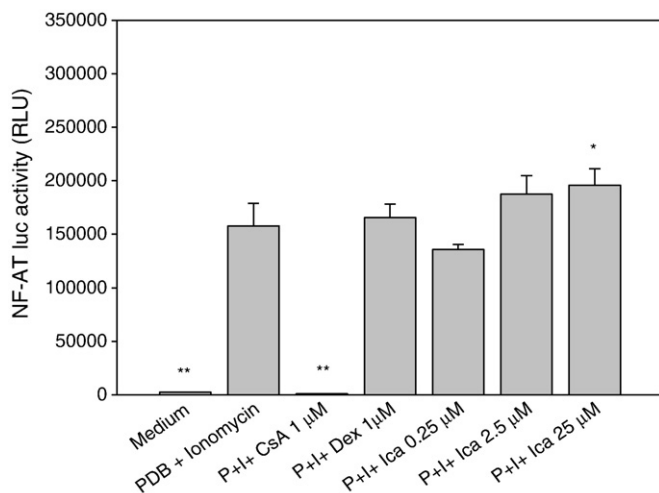


Fig. 8. Icaritin increased NF-AT luciferase activity in T cells. NF-AT constructed Jurkat T cells were cultured and treated as described in Methods. NF-AT gene transcription was measured by luciferase activity. * $P < 0.05$, as compared to PDB plus ionomycin stimulation group (P + I), ** $P < 0.01$, as compared to the P + I group.

A, as a calcineurin blocker, could inhibit IL-2 production of lymphocytes and serve as a useful therapy for transplantation rejection. Icaritin could dramatically inhibit anti-CD3 and alloantigen-induced IL-2 and IFN-γ production from 0.25 μM in this study. At this point, we assumed that icaritin might prolong the allograft skin survival via its possibilities to inhibit IL-2 and IFN-γ production like CsA. However, we found that icaritin could increase NF-AT activity compared with the blockage effect by CsA, indicating icaritin possesses different mechanisms on T cell activation compared with CsA. NF-AT is involved in both T cell activation and tolerance induction and is a well known target of CsA [27,28]. Since icaritin did not suppress NF-AT, it might not hinder T cell tolerance induction as CsA and benefit for a long time treatment.

It is interesting icaritin along with ConA can also increase IL-4 production, which indicates that icaritin might exhibit effects by shifting T cell response. In addition, in the alloantigen-induced proliferation and Th1/Th2 cytokines production experiments, we found icaritin could inhibit IL-4 production but the amount of IL-4 increases with increasing doses of icaritin. Considering that ConA stimulation only needs the TCR signal, while anti-CD3 and alloantigen stimulations also need co-stimulatory signals, we think the effect of icaritin on

IL-4 production under T cell activation is more complicated. The promotion of NF-AT might contribute to the increase of IL-4 production after ConA stimulation [34]. Th2 cells were reported to inhibit the immune diseases progression [32], we thus assume that icaritin might also inhibit immune activation by shifting Th1/Th2 responses.

Icaritin was previously reported as an estrogen agonist [12], presuming it probably had estrogen-like effects to modulate Th1/Th2 balance, affect co-stimulatory factors of APC, reduce effector T cells, induce regulatory T cells and protect Th1 dominated autoimmune diseases [35–40]. Also, there are possibilities for it to suppress T cell activation by suspending ERK and Jak-STAT pathway or inducing apoptosis based on its pharmacological effects independent on estrogen [8–11]. Recently, calpastatin, even slightly increasing T cell proliferation, was shown to extend skin graft survival by slowing down T cell migration, which provides another idea to explore its mechanism on transplantation protection [41].

In conclusion, we found icaritin could exhibit immune suppressive effects. It showed inhibitory effects at low concentration both *in vitro* and *in vivo*. It also exhibited different mechanism on T cell activation in contrast to CsA. Further efforts are expected to investigate how it works on the immune inhibition.

Acknowledgements

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