International Immunopharmacology 9 (2009) 978–983

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Saikosaponin a inhibits the proliferation and activation of T cells through cell cycle arrest and induction of apoptosis

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ARTICLE INFO

Article history:
Received 31 January 2009
Received in revised form 1 April 2009
Accepted 6 April 2009

Keywords:
Saikosaponin a
T cell
Immunosuppressive
Cell cycle arrest
Apoptosis

ABSTRACT

In the present study, we aimed at examining the immunosuppressive activity of saikosaponin a, a triterpene saponin derived from Bupleurum falcatum L. (Umbelliferae), and the underlying mechanisms. Saikosaponin a significantly inhibited the proliferation and activation of T cells activated by concanavalin A (Con A) in a concentration-dependent manner. Additionally, it potently suppressed Con A-stimulated IL-2, IFN-γ and TNF-α production in mouse T cells. Saikosaponin a also caused G0/G1 arrest of activated T cells through down-regulating the protein levels of CDK6 and Cyclin D3 and up-regulating the protein level of $p27^{kip}$. Furthermore, the compound dose-dependently induced apoptosis of Con A-activated T cells rather than those non-activated, as determined by Annexin V/PI staining. Besides, it induced a remarkable collapse of mitochondrial membrane potential and caused significant release of cytochrome c from mitochondria to cytosol. In summary, these results suggest that the G0/G1 arrest as well as the induction of apoptosis via mitochondrial pathway are involved in the immunosuppressive activity of saikosaponin a against activated T cells. This may herald a novel approach for further studies of saikosaponin a as a candidate for the treatment of inflammatory and autoimmune diseases.

1. Introduction

Saikosaponin a is a triterpene saponin derived from the medical plant, Bupleurum falcatum L. (Umbelliferae), which exhibits a variety of pharmacological activities including anti-inflammatory, immunomodulatory, and anti-bacterial activities [1–3]. The anti-inflammatory properties of saikosaponins have been demonstrated by their inhibition of mouse ear and paw edema induced by phorbol 12-myristate 13-acetate in vivo, and reduction of cyclooxygenase and lipoxygenase production in vitro [1]. The immunoregulatory action of saikosaponin a includes the suppression of anti-sheep erythrocytes plaque-forming cells responses [2]. Recently, antiallergic [4], antitumor [5,6], and antioxidant [7] activities of saikosaponin a have also been reported in laboratory studies. In addition, owing to the high content and potent pharmacological activity, saikosaponin a is also considered as a marker for quantity control of the Bupleuri radix and the traditional Chinese medicines prescribed with Bupleuri radix [8,9].

T cells play a pivotal role in immune response. Excessive T-cell proliferation and activation has been implicated in the pathogenesis of a variety of autoimmune diseases, such as hepatitis, multiple sclerosis, and rheumatoid arthritis [10–12]. Concanavalin A (Con A) is a well-known T cell mitogen, which triggers polyclonal T cell activation and the production of cytokines in vitro. On one hand, the activation of T cells involves the inductions of several cell surface molecules such as CD69 (a very early activation antigen) and CD25 (IL-2 receptor alpha chain) that participate in cell proliferation and correlate with the degree of immune activation. A number of studies have illustrated that the increased CD69 and CD25 expression correlates with some cell-mediated autoimmune and inflammatory diseases [13,14]. On the other hand, once T cell is activated, G1 cyclins (D-type cyclins) and cyclin-dependent kinase (CDK) 6/4 are induced and CDK inhibitor $p27^{kip}$ protein is reduced. All these changes regulate quiescent cell entry into the cell cycle [15]. Concomitant with the entry of cells into the cell cycle, DNA synthesis and cell division are initiated.

Considering the variety of pharmacological activities of saikosaponin a, we wonder whether saikosaponin a exerts potent immunosuppressive activity. In the present study, we provide evidence, for the first time, to demonstrate the mechanism of immunosuppressive activity of saikosaponin a. Our results reveal that the G0/G1 phase arrest as well as the induction of apoptosis via mitochondrial pathway are involved in the anti-proliferative activity of saikosaponin a against T cells. This may herald a novel approach for further studies of saikosaponin a as a candidate for the treatment of inflammatory and autoimmune diseases.

2. Materials and methods

2.1. Animals

Six-to-eight-week-old female BALB/c mice were purchased from Experimental Animal Center of Yangzhou University (Yangzhou, China). Animal welfare and experimental procedures were carried out strictly in accordance with the Guide for the Care and Use of Laboratory Animals

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doi:10.1016/j.intimp.2009.04.006

(The Ministry of Science and Technology of China, 2006) and the related ethical regulations of our university. All efforts were made to minimize animals' suffering and to reduce the number of animals used.

2.2. Cells and reagents

Mouse $CD3^+$ T cells from lymph node of BALB/c mice were purified using the Pan T cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) via magnetic cell separation with more than 98% purity. T cells were incubated in RPMI 1640 medium supplemented with 100 U mL^{-1} of penicillin, 100 μg mL^{-1} of streptomycin and 10% fetal calf serum under a humidified 5% (v/v) CO_2 atmosphere at 37 °C. Saikosaponin a (purity>98%, obtained from the National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China) is dissolved at a concentration of 0.05 mol/L in 100% DMSO as a stock solution, stored at -20 °C, and diluted with medium before each experiment. The final DMSO concentration did not exceed 0.1% throughout the study (all the control groups are composed of 0.1% DMSO). Other drugs and reagents used in this study are as follows: 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Sigma Chemical Co., St. Louis, MO); Cyclosporin A (CsA, Sandoz Ltd, Basel, Switzerland); injection dexamethasone sodium phosphate (Dex, Nanjing 3rd pharmaceutical factory, Nanjing, China); 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzimida-zolcarbocyanine iodide (JC-1) (Molecular Probes, Eugene, OR); Concanavalin A (Con A, Sigma Chemical Co., St. Louis, MO); Mouse anti-CD3 (NA/LE) and mouse anti-CD28 (NA/LE) (BD PharMingen, San Diego, CA); Annexin V-FITC/PI Kit (Jingmei Biotech, Nanjing, China); anti-cytochrome c oxidase subunit IV (COX-IV), anti-cytochrome c (Cyt c), anti-Cyclin D3, anti-$p27^{kip}$, anti-CDK6, anti-Actin and anti-α Tubulin (Santa Cruz Biotechnology, Santa Cruz, CA); peroxidase-labeled anti-mouse/rabbit antibody (KPL, Gaithersburg, ML).

2.3. MTT proliferation assay

Lymph node cell isolated from BALB/c mice were cultured in 96-well plates at a density of 3×10^5 cells/well in RPMI 1640 medium (0.2 ml) and stimulated with 5 μg/mL of concanavalin A (Con A) in the presence/absence of saikosaponin a for 72 h at 37 °C in 5% CO_2/air, then the cell viability was assessed by MTT assay.

2.4. [^{3}H] uptake proliferation assay

Lymph node cell isolated from BALB/c mice were cultured in 96-well plates at a density of 3×10^5 cells/well in RPMI 1640 medium (0.2 ml) and stimulated with Con A (5 μg/mL) or anti-CD3 (10 μg/mL) plus anti-CD28 (1 μg/mL) in the presence/absence of saikosaponin a for 66 h at 37 °C in 5% CO_2/air, then they are incubated with 0.5 μCi/well of [methyl-^{3}H] thymidine (ICN Pharmaceuticals, Costa Mesa, CA) for 6 h before harvesting as previously reported [16]. The cells are harvested onto filter paper and the uptake was measured as counts per minute (c.p.m.) by a liquid scintillation counter.

2.5. Analysis of CD69 and CD25 cell surface expressions

The expressions of cell surface molecules in T cell cultures were evaluated by flow cytometry. T cells (5×10^5) were stimulated with 5 μg/ml of Con A with the addition of saikosaponin a simultaneously. The surface expressions of CD69 and CD25 were assessed after 24 h of culture, respectively. At the end of the culture period, the harvested cells were washed twice with buffer. Cells were stained with specific antibodies for 30 min at 4 °C in the dark. Cells were then washed with buffer to remove the excess stains and analyzed. Samples were analyzed in a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) using CellQuest software.

2.6. Enzyme-linked immunosorbent assay

The levels of IL-2, IFN-γ and TNF-α produced in activated mouse T cells were measured using the mouse enzyme-linked immunosorbent assay (ELISA) system (R&D systems, Minneapolis, MN).

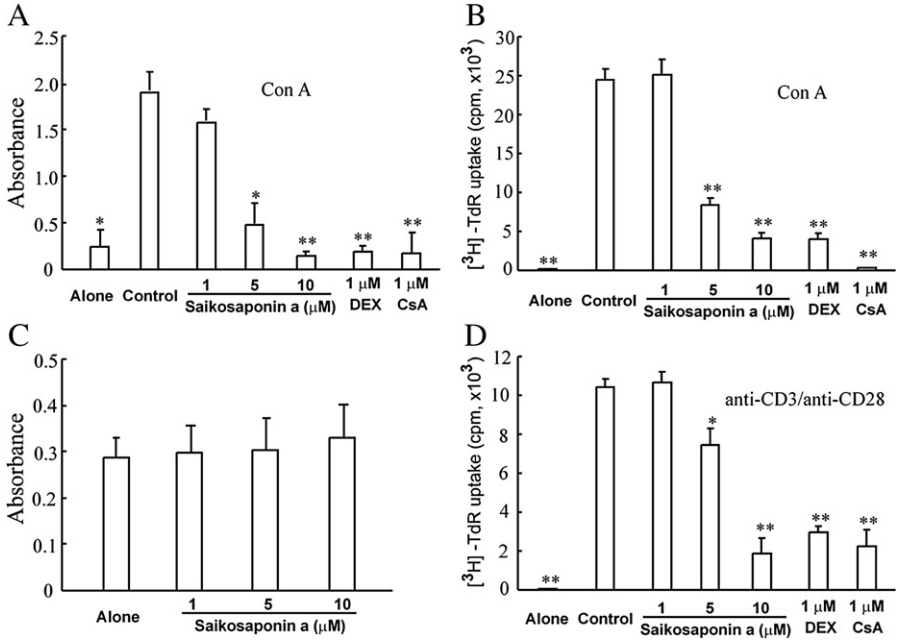

Fig. 1. Effects of saikosaponin a on T cell proliferation activated by Con A. Lymph node cell isolated from BALB/c mice were cultured in 96-well plates at a density of 3×10^5 cells/well in RPMI 1640 medium and stimulated with 5 μg/mL of Con A for 72 h at 37 °C in 5% CO_2/air, then the cell proliferation was assessed by MTT assay (A) and [^{3}H]-thymidine uptake assay (B). (C) Lymph node cell isolated from BALB/c mice were seeded in 96-well plates at a density of 3×10^5 cells/well in RPMI 1640 medium and co-cultured with various concentrations of saikosaponin a for 24 h at 37 °C in 5% CO_2/air, then the cell viability was assessed by MTT assay. (D) Lymph node cell isolated from BALB/c mice were cultured in 96-well plates at a density of 3×10^5 cells/well in RPMI 1640 medium and stimulated with anti-CD3 (10 μg/mL) and anti-CD28 (1 μg/mL) for 72 h at 37 °C in 5% CO_2/air, then the cell proliferation was assessed by [^{3}H]-thymidine uptake assay. Data represent the mean ± SEM of three independent experiments in triplicate. *P<0.05, **P<0.01 vs Control.

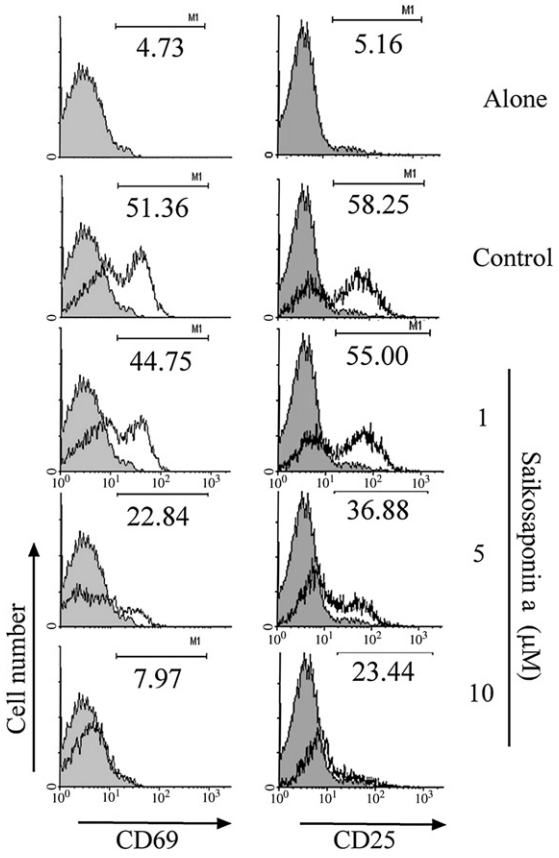

Fig. 2. Effects of saikosaponin a on CD69 and CD25 cell surface expressions in activated mouse T cells. The purified T cells from lymph node of BALB/c mice were stimulation with 5 μg/ml of Con A in the absence or presence of saikosaponin a for 24 h in vitro. Then cells were harvested and CD69 and CD25 expressions were determined by flow cytometry. Histogram data shown here are one of three independent experiments.

2.7. Cell cycle assay

To examine the effect of saikosaponin a on cell cycle distribution, T cells from lymph nodes of BALB/c mice were treated with or without 1, 5, 10 μM of saikosaponin a for 24 h in the presence of Con A (5 μg/ml), and then collected and washed with cold PBS and fixed with 70% ethanol at 4 °C overnight. Then, the fixed cells were washed with PBS and stained with 50 μg/ml of propidium iodide (PI) containing 100 μg/ml of RNase A and 1% TritonX-100 in the dark at room temperature for 45 min. The DNA contents of the cells were analyzed with Modfit software (Becton Dickinson, San Jose, CA, USA).

2.8. Western blot

After the incubation, T cells are harvested and lysed. Proteins are quantified using a BCA protein assay kit (Pierce, Rockford, IL) according to the manufacturer's specifications. Then proteins were separated by SDS-PAGE and electrophoretically transferred onto polyvinylidene fluoride membranes. The membranes were probed with antibodies overnight at 4 °C, and then incubated with a HRP-coupled secondary antibody. Detection was performed using a LumiGLO chemiluminescent substrate system (KPL, Guildford, UK).

2.9. Cell apoptosis assay

Cell apoptosis was determined by Annexin V-FITC (fluorescein isothiocyanate)/PI (propidium iodide) staining as previously reported [17]. Samples were analyzed by FACSCalibur flow cytometer.

2.10. Cell mitochondrial membrane potential assay

Mouse T cells isolated from lymph node of BALB/c mice were treated with or without 1, 5, 10 μM of saikosaponin a for 12 h in the presence of Con A (5 μg/ml). Then cells were harvested and the disruption of mitochondrial transmembrane potential was measured using JC-1 staining by flow cytometry as previously reported [18].

2.11. Subcellular fractionation

The proteins in the purified mouse T cells were separated into cytosolic and mitochondrial fractions using the ProteoExtract Cytosol/Mitochondria Fractionation Kit (Merck Bioscience, Bad Soden, Germany) according to the procedures provided by the manufacturer. To check the selectivity of proteins from subcellular fractionation, Tubulin and COX-IV were used as marker proteins representing the cytosolic and mitochondrial fractions, respectively.

2.12. Statistical analysis

Data are expressed as mean ± SEM. Student's *t* test is used to evaluate the differences between various experimental and control groups. *P* values less than 0.05 were considered significant.

3. Results

3.1. Effects of saikosaponin a on T cell proliferation

As shown in Fig. 1A, saikosaponin a significantly inhibited T cell proliferation induced by Con A in a concentration-dependent manner by MTT assay: more than 70% at 5 μM and almost completely at 10 μM. The same result was also seen in [^{3}H]-thymidine uptake assay. Saikosaponin a significantly inhibited Con A-stimulated [^{3}H]-thymidine incorporation of T cells in a concentration-dependent manner (Fig. 1B). The positive control dexamethasone (DEX) and cyclosporine A (CsA) also remarkably inhibited T cell proliferation. It is important to note that, saikosaponin a at the concentrations mentioned above did not affect T lymphocyte's viability by MTT uptake assay (Fig. 1C). These results also indicated that the immunosuppressive activity of saikosaponin a observed here, at concentrations which are up to 10 μM, is not caused by its cytotoxicity. Moreover, saikosaponin a also significantly inhibited anti-CD3/anti-CD28-stimulated [^{3}H]-thymidine incorporation of T cells in a concentration-dependent manner (Fig. 1D).

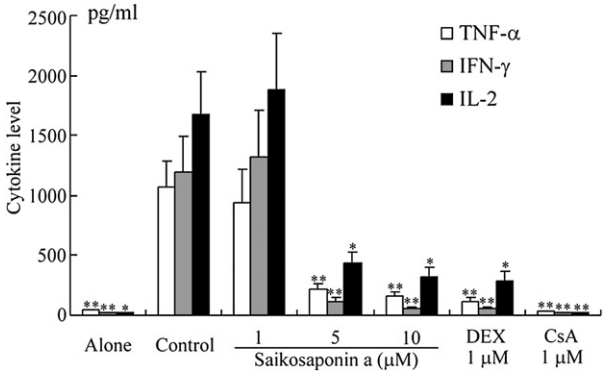

Fig. 3. Effect of saikosaponin a on the production of proinflammatory cytokines in activated mouse T cells. The purified T cells from lymph node of BALB/c mice were stimulated with Con A (5 μg/ml) for 24 h in the absence or presence of saikosaponin a (1, 5 and 10 μM). The concentrations of the cytokines in cell culture supernatants were determined by ELISA. Data represent the mean ± SEM of three independent experiments in triplicate. *P<0.05, **P<0.01 vs Control.

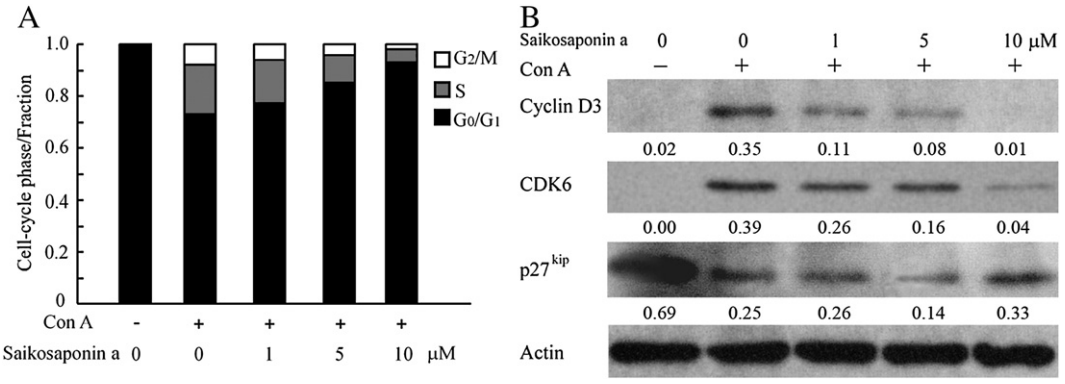

Fig. 4. Effect of saikosaponin a on the cell cycle in activated mouse T cells. The purified T cells from lymph node of BALB/c mice were treated with or without 1, 5, 10 μM saikosaponin a for 24 h in the presence of 5 μg/ml Con A. On one hand, the cells were stained with propidium iodide (PI) and analyzed by flow cytometry (A). On the other hand, the cells were harvested and lysed. The expressions of cell cycle regulators were analyzed by Western blotting (B). All data are one of three different experiments.

3.2. Effects of saikosaponin a on CD69 and CD25 cell surface expressions in activated mouse T cells

CD69 and CD25 induction can be triggered by Con A which acts as a T cell mitogen to interact with the T cell receptor (TCR)/CD3 complex in T cells. It was shown that CD69 and CD25 cell surface expressions were up-regulated in mouse T cells after 24 h incubation with Con A (5 μg/ml), while saikosaponin a mediated a potent inhibitory effect on CD69 and CD25 expressions in Con A-treated mouse T cells in a dose-dependent manner (1–10 μM) (Fig. 2).

3.3. Effect of saikosaponin a on the production of proinflammatory cytokines in activated mouse T cells

To examine the effect of saikosaponin a on the production of proinflammatory cytokines such as IL-2, IFN-γ and TNF-α, ELISA was performed to measure the level of the cytokines in the culture supernatant of activated T cells. Our results show that the stimulation of mouse T cells with Con A resulted in the considerable production and secretion of IL-2, IFN-γ and TNF-α into the culture medium. Significant reduction of these proinflammatory cytokines was found in activated T cells treated with saikosaponin a (5 and 10 μM) (Fig. 3). The inhibitory ratio of saikosaponin a (10 μM) on these cytokines is almost equivalent to positive control dexamethasone (1 μM).

3.4. Effect of saikosaponin a on the cell cycle in activated mouse T cells

As shown in Fig. 4A, Con A (5 μg/ml) stimulation resulted in the progression into the S and G2/M phase and notable DNA synthesis. With flow cytometry analysis, the propidium iodide-stained cells showed a significant arrest in the G0/G1 phase of the cell cycle following saikosaponin a (1–10 μM) treatment.

Cell entry into the cell cycle and progression through the G1 phase is dictated by the presence of D-type cyclins (cyclin D1, cyclin D2 and cyclin D3) in complex with the G1-phase cyclin-dependent kinases CDK4/CDK6 [19,20]. It has been reported that cyclin D2/3, CDK6 and CDK inhibitor $p27^{kip}$ play major roles in cell cycle of T cells [15]. For further demonstration of the effect of saikosaponin a on cell cycle, we investigated the influence of saikosaponin a on the protein levels of cyclin D3, CDK6 and $p27^{kip}$ in purified T cells. Consistent with the

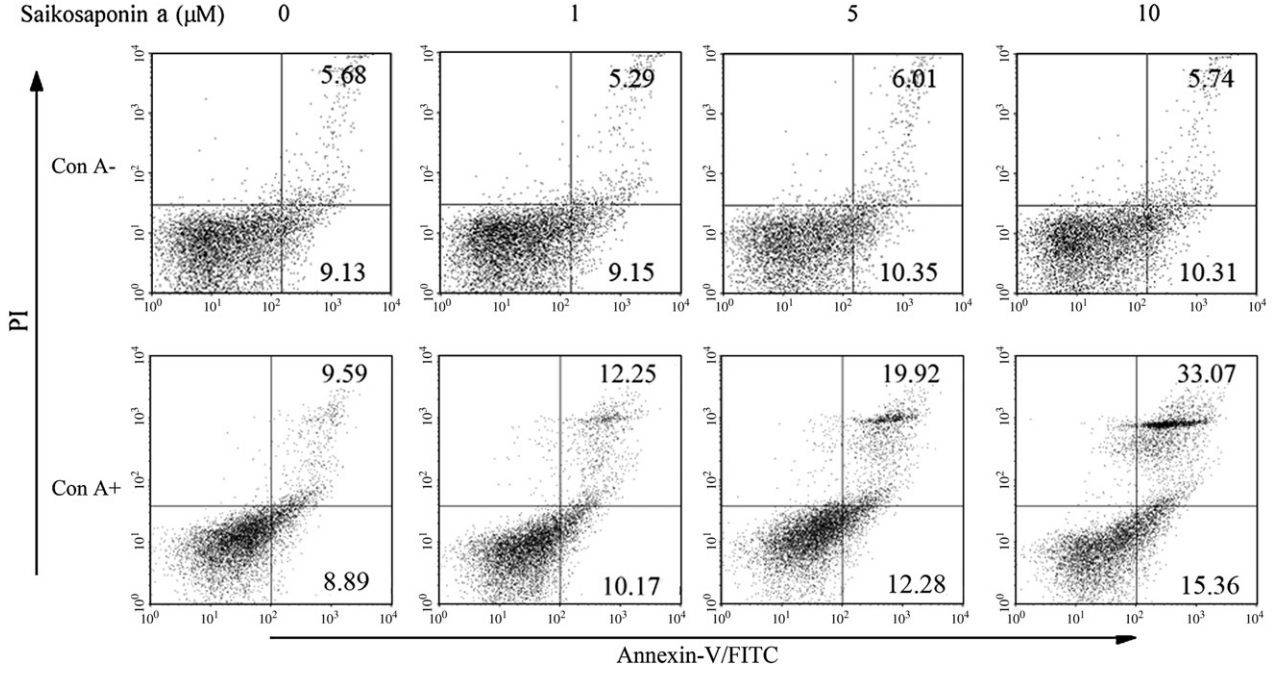

Fig. 5. Effect of saikosaponin a on the cell apoptosis in Con A-activated mouse T cells and naïve T cells. The purified T cells from lymph node of BALB/c mice were treated with or without 1, 5, 10 μM of saikosaponin a for 24 h in the presence or absence of 5 μg/ml of Con A. Then the cells were assayed for the percentages of the Annexin V-positive populations. The data shown here are one of three different experiments.

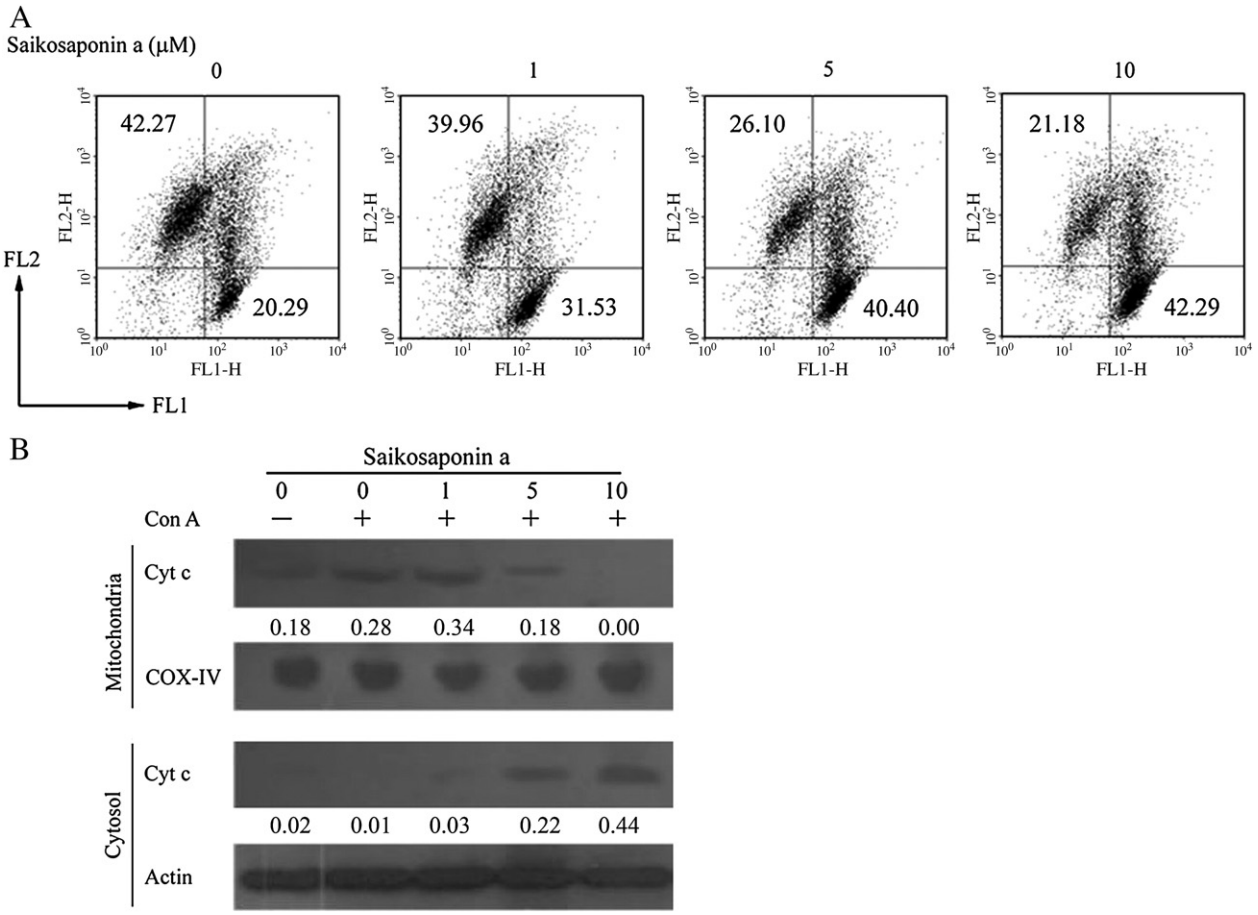

Fig. 6. Effect of saikosaponin a on the mitochondrial membrane potential and cytochrome c release in activated mouse T cells. The purified T cells from lymph node of BALB/c mice were treated with or without 1, 5, 10 μM of saikosaponin a for 24 h in the presence of 5 μg/ml of Con A. On one hand, the cells were harvested and the disruption of mitochondrial transmembrane potential was measured using JC-1 staining by flow cytometry (A). Dots on the lower side indicate cells with lower membrane potential. On the other hand, the cells were harvested and separated into cytosolic and mitochondrial fractions using the commercial fractionation kit. The expressions of cytochrome c in cytosol and mitochondria were analyzed by Western blotting (B). The data shown here are one of three different experiments.

observations on cell cycle distribution, saikosaponin a dramatically inhibited the increase of cyclin D3 and CDK6 and suppressed the degradation of $p27^{kip}$. The results showed that saikosaponin a influenced the cell cycle regulatory molecules of G1 phase and blocked cell cycle progression through G1/S transition (Fig. 4B).

3.5. Effect of saikosaponin a on the cell apoptosis in activated mouse T cells

Mouse T cells were treated with or without different concentrations of saikosaponin a for 24 h in the presence or absence of 5 μg/ml of Con A. As shown in Fig. 5, the supravital exposure to propidium iodide and Annexin V labelling demonstrated that saikosaponin a (1–10 μM) significantly induced both apoptosis (Annexin V^+ PI^- staining) and necrosis (Annexin V^+ PI^+ staining) of Con A-activated T cells in a dose-dependent manner. However, saikosaponin did not affect naive T lymphocyte's viability by Annexin V-FITC/PI staining assay. Together with the result of MTT assay in Fig. 1C, these findings suggest that the immunosuppressive activity of saikosaponin a observed in the present study is not caused by its cytotoxic effect.

The mitochondrial apoptosis pathway is one of the critical pathways of apoptosis. Alterations in mitochondrial membrane potential were examined after saikosaponin a treatment. As shown in Fig. 6A, a reduction in mitochondrial membrane potential was clearly seen in saikosaponin a-treated groups in a dose-dependent manner. In addition, mitochondrial protein and cytosolic protein was isolated from saikosaponin a-treated activated T cells, respectively, then these proteins were subjected to Western blotting. Equal protein loading was confirmed by immunodetection of COX-IV for mitochondrial protein or tubulin for cytosolic protein. As shown in Fig. 6B, saikosaponin a greatly increased the cytosolic cytochrome c and notably decreased the mitochondrial cytochrome c as compared with the control group.

4. Discussion

T lymphocytes play a pivotal role in the pathogenesis of cell-mediated autoimmune diseases and the chronic inflammatory disorders [12,21]. Previous studies suggested that saikosaponin a could suppress antibody formation against heterologous erythrocytes [2]. Recently, Leung et al. have demonstrated that saikosaponin a analog saikosaponin d inhibits T cell activation through the modulation of PKCtheta, JNK, and NF-kappaB transcription factor [22]. However, the effect of saikosaponin a on T lymphocytes is still largely unknown. Therefore, in the present study, we aimed to clarify the actions of saikosaponin a on T cells and investigate the underlying mechanisms mediating its immunosuppressive activity.

Previously, Tang et al developed a high-performance liquid chromatographic method with UV detection to determine saikosaponin a in rat plasma samples in a pharmacokinetic study [23]. They found that the concentration of saikosaponin a in rat plasma following 15 mg/kg intravenous dose of the compound reached a peak at about 3.3 μM, which just fell within the range of the drug concentrations (1–10 μM) used in the present study. We first examined the effect of saikosaponin a on T cell mitogen Con A-induced T-cell proliferation in vitro. As a result, the proliferative response induced by Con A was dose-dependently

suppressed by saikosaponin a at non-cytotoxic concentrations. Moreover, as to T-cell activation, saikosaponin a also remarkably inhibited Con A-induced increase of the expressions of CD25 and CD69, which were usually considered as the T-cell activation marker molecules. These results indicated that saikosaponin a inhibited the mitogenic proliferation and activation of T cells. Further studies then elucidated the action of saikosaponin a on cytokine production of T cells. Consistent with the observations above, saikosaponin a dose-dependently inhibited Con A-induced production of IL-2, IFN-γ and TNF-α in purified T cells from normal mice. These findings indicated that saikosaponin a exerted immunosuppressive effect through its inhibition of T-cell proliferation, activation, and cytokine production.

To elucidate some of the underlying mechanisms of saikosaponin a against T cells, we further examined the effect of saikosaponin a on cell cycle of T cells. PI staining data indicated that saikosaponin a inhibited T-cell cycle progression, and we found that T-cell proliferation was generally controlled by saikosaponin a at the G0/G1 phases. The eucaryotic cell cycle is regulated by the periodic synthesis and destruction of cyclins that associate with and activate CDK [24]. CDK and CDK inhibitors are the key regulators of cell-cycle transitions. In mammalian cells, CDK4, CDK6 and associated CDK inhibitor $p27^{kip}$ control the G1 to S phase transition [15,25]. Cell progression through G1 to S transition was markedly inhibited by saikosaponin a, which was correlated with a saikosaponin a-mediated reduction of the protein levels of G1-phase cell cycle molecules (cyclin D3 and CDK6) and an increase in CDK inhibitor $p27^{kip}$. These findings suggested that saikosaponin a influenced T-cell activation and inhibited T-cell cycle progression in vitro.

Apoptosis is an essential mechanism used to eliminate activated T cells during the shutdown process of excess immune responses and maintain proper immune homeostasis, while deficient apoptosis of activated T cells is associated with a wide variety of immune disorders [26,27]. One of the typical characters of the apoptotic cells is a phosphatidylserine shift toward the outer leaflet of the cell membrane [28]. Fig. 5 showed that saikosaponin a significantly triggered apoptosis of Con A-activated T cells rather than those un-activated naïve T cells in a dose-dependent manner, suggesting the role of apoptosis induction in the anti-proliferation mechanisms of saikosaponin a. Herein we reported that saikosaponin a, having little effect on non-activated T cells, selectively promoted apoptosis in activated T cells, which avoided the disadvantage of non-specific immunosuppression. Mitochondria are known as the bioenergetic and metabolic centers of eucaryotic cells. During the process of apoptosis, mitochondria suffer specific damage, including perturbation of mitochondrial membrane permeability. As a result, cytochrome c is released to the cytosol. Once released, cytochrome c, in interaction with apoptotic protease activating factors, initiates the activation of caspase-9 that leads to the subsequent apoptosis [29]. To investigate how saikosaponin a induces apoptosis of activated T cells, we further observed several cellular events relating to the induction of apoptosis. Our result showed that saikosaponin a dose-dependently disrupted the mitochondrial membrane potential, and caused release of cytochrome c from mitochondria to cytosol. All these findings indicated the involvement of the mitochondrial pathway in the apoptosis induction of saikosaponin a.

Here, we have demonstrated that saikosaponin a inhibited T-cell activation and proliferation. The immunosuppressive mechanism of saikosaponin a correlated with the G0/G1 phase arrest and the induction of apoptosis via mitochondrial pathway in T cells. These findings extended our understanding of the immunosuppressive effect of saikosaponins. It also suggested the potential of saikosaponins as the effective candidate compounds for use in the treatment of inflammatory and autoimmune diseases.

Acknowledgments

This work was supported by funds from the National Natural Science Foundation of China (Nos. 30701095, 30730107).

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