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ORIGINAL ARTICLE

Effect of Diosgenin on T-Helper 17 Cells in Mice with Collagen-Induced Arthritis

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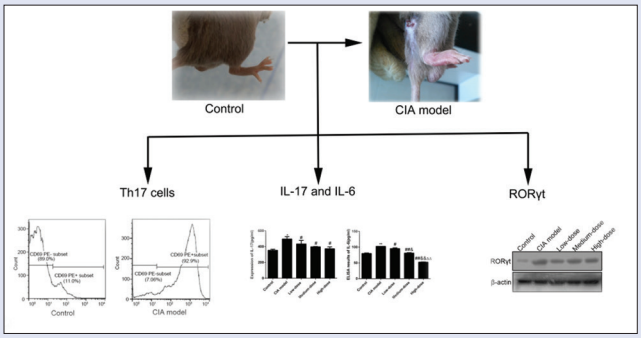
ABSTRACT

Background: Diosgenin, obtained from *Dioscorea nipponica* Makino, possesses anti-inflammatory properties. **Objective**: The objective of this study is to explore the therapeutic effects of diosgenin against collagen-induced arthritis (CIA) in DBA/1J mice. **Materials and Methods:** The CIA model was established using chicken type II collagen-immunized DBA1/J mice (0.1 mL/mouse), with the same stimulus repeated on day 21. Next, the CIA mouse model was selected, and inguinal lymph nodes were excised to obtain a single-cell suspension. Using CCK-8, control group, model group, and high-/medium-/low-dose diosgenin group were set based on drug toxicity. Next, the ratio of T-helper 17 cells (Th17) to $CD3^+$ T cells was analyzed using flow cytometer. The levels of interleukin 17 (IL)-17 and IL-6 were determined by the enzyme-linked immunosorbent assay. The expression of RORγt was determined using the reverse-transcription quantitative real-time polymerase chain reaction and Western blot analysis. **Results:** An *in vitro* inhibitory rate of ≥ 70% was set as the cutoff value to select the high (25 μmol/L), medium (12.5 μmol/L), and low (6.25 μmol/L) doses of diosgenin. The Th17 cell ratio in $CD3^+$ T cells, the levels of IL-17 and IL-6, and mRNA and protein expression of RORγt showed significantly increased tendency in the model group compared with the control group ($P < 0.01$ and/or $P < 0.05$). Diosgenin significantly decreased the Th17 cell ratio in $CD3^+$ T cells, IL-17 and IL-6 levels, and RORγt expression when compared with the model group ($P < 0.01$ and/or $P < 0.05$). **Conclusion:** Diosgenin exhibited an antiarthritic effect in CIA mice by downregulating the differentiation of Th17 cells.

Key words: Collagen-induced arthritis mice, diosgenin, interleukin-17, interleukin-6, RORγt, T-helper 17 cell

SUMMARY

- Diosgenin exhibited an antiarthritic effect in CIA mice by downregulating the differentiation of Th17 cells.
- Diosgenin significantly decreased the Th17 cell ratio in CD3+ T cells, IL-17 and IL-6 levels, and RORγt expression.



Abbreviations used: Th17: T helper 17 cells, IL: interleukin, CIA: collagen-induced arthritis, RA: Rheumatoid arthritis, AI: arthritis index, OD: optical dentistry, PMA: phorbol-12-myristate-13-acetate, BFA: brefeldin A.

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INTRODUCTION

Rheumatoid arthritis (RA) is a chronic autoimmune disease, whose pathological characteristics are progressive articular damage, loss of joint function, and high possibility of disability.[1,2] It is estimated that approximately 1% of the adults suffer from RA; however, its pathogenesis remains unclear.[3,4] Despite the rapid advances in RA treatment, new drugs with high efficacy but few long-term adverse effects are still required.[5-7]

Notably, multiple molecules and cells are associated with the development of RA. Autoimmune injury mediated by abnormal T-cell could influence RA pathological process.[1,8] $CD4^+$ T-helper (Th) cells, key constituent of effector T-cells, include Th1, Th2, regulatory T-cells (Tregs), and Th17 cells, which are well-known contributors for RA.[9] Debbie *et al.* indicated that reduction of Th17 cells and interleukin 17 (IL)-17 secretion suppressed the pathological symptoms of RA.[10] Ye *et al.* suggested that inhibiting the proliferation of Th17 cells and the yield of IL-17 could alleviate RA.[11] Furthermore, Th17 cells and proinflammatory cytokines regulate osteoclastogenesis in the development of RA.[12] In addition, the imbalance in the ratio of Treg/Th17 cells is involved in the etiological mechanism of RA.[13]

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These findings demonstrate that Th17 cells act as a target for the anti-inflammatory treatment of RA.

Dioscorea nipponica Makino is a Chinese herb belonging to the family *Dioscoreaceae*, commonly used to treat low back and leg pain and RA.[14] Diosgenin, (3-*O*-α-l-rhamnopyranosyl-[1,4]-[α-l-rhamnopyranosyl-(1,2)]-β-d-glucopyranosyl-diosgenin), is one of the most important steroidal saponins extracted from *D. nipponica*. It shows hypolipidemic, antioxidant, and antineoplastic effects.[15-18] It has been reported that diosgenin may be useful in cuing inflammatory diseases.[15,19,20] Diosgenin has shown efficacy against osteoarthritis by effectively inhibiting the expression of inflammatory mediators, including nitric oxide, prostaglandin E2, inducible nitric oxide synthase, and cyclooxygenase-2.[21] However, according to our knowledge, diosgenin has been rarely used in the treatment of RA. In the current study, the function of diosgenin in Th17 cells was preliminarily explored in collagen-induced arthritis (CIA) mouse model.

MATERIALS AND METHODS

Preparation of diosgenin and interleukin-2 solution

Diosgenin (Zelang Biotech, Nanjing, China) was dissolved in 10 mL ethanol as a 20 mM stock solution and stored at −20°C until utilization. Prior to the experiment, fresh agents were prepared from the stock solutions of diosgenin. The stock solution of IL-2 (Hoffman LaRoche) was prepared by dissolving 20 μg of IL-2 in 40 μL ddH_2O, and the volume was made up to 1000 μL with 960 μL phosphate buffer solution (5% trehalose) to obtain a final concentration of 100 units/mL.

Establishment of collagen-induced arthritis model

A total of 75 DBA/1J mice (7–8 weeks of age) were obtained from the SLAC Laboratory Animal Company (Shanghai, China; license number: SCXK (Hu) 2012-0002) and were housed under specific pathogen-free conditions for a week (5 mice per cage) The indoor temperature was kept at 20°C ± 2°C, and humidity was maintained at 55% ± 5%. Animal procedures were authorized by the Institutional Animal Care and Use Committee of Chengde Medical College. The ethics approval number is CDMULAC-20160904-001.

After 1 week of adaptation, 6 mice were randomly chosen for the normal control group and the others were used to establish the CIA model. First, chicken type II collagen 2 mg/mL (Sigma) was equally mixed with the Freund's complete adjuvant (Sigma) containing the bacillus Calmette-Guérin vaccine (2.5 mg/mL) via repeated aspiration. Then, the emulsified collagen (0.1 mL per mice) was intracutaneously injected at the tail base. On day 21, a repeat immune stimulation was performed using the above method. The model was confirmed on day 28 after the first injection. In the case of the control group (n = 6), an equal volume of PBS was injected at the mouse tail base instead of type II collagen.

Collagen-induced arthritis model confirmation

Following the injection, mouse characteristics such as weight, coat color, diet, and redness of joints were recorded. Moreover, the thickness of the hind paw was recorded once in every 3 days for the first injection. Arthritis was estimated based on the following criteria: (I) score 0 = no swelling; (II) score 1 = slight swelling at toe joints; (III) score 2 = swelling at toe and foot joints; (IV) score 3 = swelling at paw and foot joints, with swelling below the ankle; and (V) score 4 = swelling of paws, foot, and ankles. The arthritis index (AI) value is the sum of all four limb swelling scores (0–16). A higher AI value indicates more severe joint symptoms, and the criteria of a successful CIA model was an AI ≥4.[1,22]

Isolation and culture of lymphocytes

After the induction of CIA, lymphocytes were isolated from the inguinal lymph nodes of CIA mice for further investigation. Briefly, mice were anesthetized with an intraperitoneal injection of pentobarbital (50 mg/kg) and euthanized by rapid cervical dislocation. Next, a single-cell suspension was prepared by gently pressing the inguinal lymph nodes using a 200-mesh stainless steel mesh and maintained in RPMI 1640 (Gibco) medium with 10% fetal bovine serum (Gibco) and 1% penicillin and streptomycin (Haoyang Biotech) at 37°C with 5% CO_2. The cell concentration was measured and adjusted with 0.4% trypan blue (Sigma).

Detection of cytotoxicity

CCK-8 (Dojindo) was used to detect the cytotoxicity of diosgenin in lymphocytes. The lymphocytes were divided into seven groups and the final lymphocyte density of CIA mice was adjusted to 2×10^6 mL^{-1}. RPMI 1640 medium was used as the blank control, cells maintained in RPMI 1640 without diosgenin were used as controls, and cells treated with diosgenin (3.125, 6.25, 12.5, 25, and 50 μmol/L, respectively) were considered as the experimental group. Cells in seven groups were seeded into 96-well plates in sequence. After culturing for 44 h, the cells in each well were mixed with 20 μL of the CCK-8 reagent and cultured for 4 h. Microplate reader (Tecan Sunrise, Männedorf, Switzerland) was utilized to assess the optical dentistry (OD) at 450 nm. Finally, the relative survival rate of lymphocytes was calculated using the formula: survival rate = (1− [experimental OD − blank control OD]/[control OD − blank control OD]) × 100%. The concentration of the drug at which the survival rate was more than 70% was selected for the subsequent experiments.

Cell proliferation assay

RPMI 1640 medium (blank control group), lymphocytes in RPMI 1640 (control group), lymphocytes in RPMI 1640 treated with diosgenin (3.125, 6.25, 12.5, 25, and 50 μmol/L, respectively) (experimental group) were prepared, and added 50 μg/mL chicken collagen type II, 100 units/mL IL-2. Then 2×10^6 mL^{-1} of lymphocytes were seeded into 96-well plates After culturing for 44 h, the cells were incubated in 20 μL CCK-8 solution for 4 h, and the OD value was detected at 450 nm with a microplate reader (Tecan Sunrise). The rate of proliferation of lymphocytes was calculated using the formula: cell inhibition rate = (1− [experimental OD − blank control OD]/[control OD − blank control OD]) × 100%.

Detection of T-helper 17 cell ratio by flow cytometry

CIA mouse lymphocytes were extracted to prepare the single-cell suspension using the method described in the previous section. Then, the acquired cells were allowed to grow and were divided into control (IL-2), model (IL-2 and chicken type II collagen), and diosgenin groups (IL-2, chicken II collagen, and high-/medium-/low-dose diosgenin). The groups were treated with 100 units/mL IL-2 and 50 μg/mL chicken type II collagen. After incubation for 48 h, five groups of cells were collected in sterile tubes, and centrifuged for 8 min at 1700 r/min. Next, 300 μL of cell suspension (containing 1×10^7 cells/mL) was added to 96-well plates (2 wells for cells obtained from each mouse). Next, 1.25 μL of phorbol-12-myristate-13-acetate/ionomycin mixture (LianheBio) and brefeldin A (BFA)/monensin mixture (LianheBio) were sequentially added and cultured for 6 h at 37°C. Four additional wells were prepared for the detection of CD69 activity without the addition of BFA/monensin mixture. Then, 100 μL of the cultured cell suspension from 96-well plates was absorbed into the flow tube. The isotypic control tube contained 1.0 μL of Armenian Hamster immunoglobulin G (IgG) (e-Bioscience);

another isotypic tube contained 1.0 μL of isotypic anti-mouse CD3e FITC (e-Bioscience) and anti-mouse CD69 PE (e-Bioscience). The test tube contained 1 μL of anti-mouse CD3e FITC (e-Bioscience) and anti-mouse CD69 PE (e-Bioscience), followed by 20 min of culturing in the dark at room temperature. The ratio of Th17 cells to $CD3^+$ T cells was evaluated using intracellular antigen anti-mouse IL-17A PE (e-Bioscience). Subsequently, the cells were utilized to conduct the flow cytometry (BD Biosciences) for the Th17 assessment.

Enzyme-linked immunosorbent assay

In this study, the supernatant from the lymphocyte culture was withdrawn to determine the levels of IL-17 and IL-6, which both were measured using commercial enzyme-linked immunosorbent assay Kit (Dakewe Biotech), and all steps follow the manufacturer's instructions.

Reverse transcription quantitative real-time polymerase chain reaction

Total cellular RNA from control, model, and diosgenin groups were obtained by homogenizing the cells using Trizol reagent (Takara) and quantified using Nanodrop 2000 (Thermo Scientific). Then, 2 μg of total RNA were subjected to synthesis of cDNA by PrimeScript™ RT reagent Kit (Takara). Using SYBR® Premix Ex Taq II (Takara), reverse transcription quantitative real-time polymerase chain reaction was conducted and reaction condition is 95°C for 30 s, 40 cycles of 95°C for 5 s and 57.5°C for 30 s, and 60°C for 30 s. Gene primers were designed and synthesized by TaKaRa Biotechnology. RORγt primer sequences are listed in Table 1. GAPDH was used as the internal control, and the relative expression of RORγt was analyzed using the $2^{-\Delta\Delta Ct}$ method.[23] The average value of three parallel experiments was taken for each sample.

Western blot

The cells from control, model, and diosgenin groups were lysed in ice-cold RIPA lysis buffer (Santa Cruz Biotech). Following this, the protein lysates were quantified using a BCA protein assay kit (Pierce). 50 μg of protein was performed to separate on (sodium dodecyl sulphate-polyacrylamide gel electrophoresis (8%), and electrotransferred onto polyvinylidene fluoride membranes (Amersham Pharmacia Biotech). The membranes were blocked with 5% skim milk in Tris-buffered saline-Tween overnight, and then incubated with polyclonal anti-RORγt antibody (1:500, Abcam) and horseradish peroxidase-labeled anti-Ig antibody conjugate (1:5000, Beyotime Biotech). Subsequently, the membranes were rinsed thrice with TBST for 10 min and visualized using the enhanced chemiluminescence kit (Millipore). Image Lab software (Bio-Rad) was used to assess the gray value of genes and the relative expression of RORγt in each group was normalized to β-actin.

Statistical analysis

Statistical comparisons were evaluated using the SPSS software version 17.0 (SPSS Inc., Chicago, IL, USA). The experimental results are presented as mean ± standard deviation. When date meets the normal distribution and homogeneity of variances, a one-way analysis of variance was used to compare between any two groups, and the Newman-Keuls Q test was performed for comparison between multiple groups. Otherwise, the Games-Howell *post-hoc* test was conducted. For all comparisons, $P < 0.05$ indicated significance.

RESULTS

Confirmation of collagen-induced arthritis model

While constructing the CIA model, the dorsal surface and tail of the animals showed the presence of ulcers of varying sizes. In addition, the coat color gradually faded, and feed consumption and weight significantly reduced over time. On day 28, the mice presented with obvious foot and paw joint swelling, as well as showed reduced activity [Figure 1]. In the model group, AI was assessed as 8.17 ± 4.19 on day 28, which was much greater than that in the control group (AI = 0, $P < 0.01$), indicating that the CIA model was successfully established.

Diosgenin inhibited the proliferation of lymphocytes after interleukin IL-2 and collagen treatment

First, the cytotoxicity of diosgenin was assessed using CCK-8 assay. According to our results, more than 70% of the lymphocytes survived after incubating the cells with diosgenin for 48 h at a concentration ranging between 3.125 and 25 μmol/L [Table 2]. This showed that diosgenin has no obvious cytotoxic effect on the lymphocytes. Moreover, treatment with diosgenin caused inhibitory effect of lymphocyte. The rate of inhibition of proliferation at 3.125, 6.25, 12.5, 25, and 50 μmol/L concentrations was 0.083, 0.215, 0.498, 0.722, and 0.905, respectively [Table 3]. Based on these results, diosgenin at 6.25, 12.5, and 25 μmol/L concentrations was set as the low-, medium-, and high-dose groups and used for the following investigations.

Diosgenin reduced the ratio of T-helper 17 in the collagen-induced arthritis model

The ratio of $CD3^+$ $CD69^+/CD3^+$ T-cells in the activation group was >90%, indicating that the lymphocytes were successfully activated [Figure 2a]. Model group promoted the production of Th17 cells in $CD3^+$ T cells as comparing with the control ($^*P < 0.05$). While, both medium-and high-dose diosgenin treatment were suggested to markedly reduce the ratio of Th17 cells in $CD3^+$ T cells compared with the model ($^\#P < 0.05$) and low-dose groups ($^\&P < 0.05$) [Figure 2b and c].

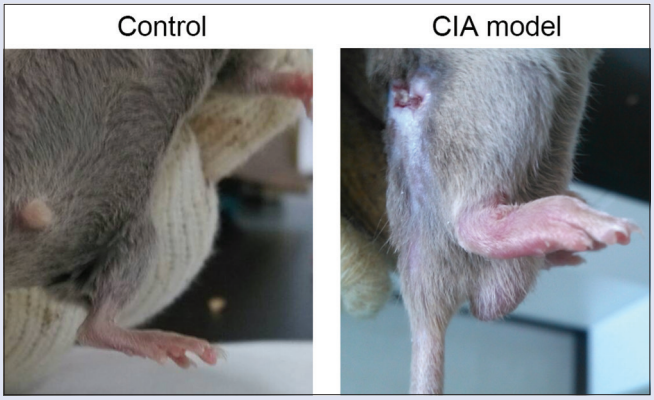

Figure 1: Construction of collagen-induced arthritis model in DBA/1J mice for 28 days

Table 1: Sequences of the primers used in this study

Primer	Sense (5′-3′)	Antisense (5′-3′)
RORγt	GCTCCATATTTGACTTTTCCCACT	GATGTTCCACTCTCCTCTTCTCTTG
GAPDH	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA

Diosgenin supressed the expression of interleukin-17 and interleukin-6 in the collagen-induced arthritis model

IL-17 and IL-6 expression levels were measured in lymphocytes isolated from the CIA model [Figure 3a and b]. Model group showed considerable increase of IL-17 and IL-6 expression levels in comparison with the controls ($^{*}P < 0.05$, $^{**}P < 0.01$). But mice treated with three diosgenin concentrations all showed lower expressions of IL-17 and IL-6 in the comparison with the model group ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$). Furthermore, IL-6 level in treatment with medium- and high-dose diosgenin indicated obviously reduced trend in the comparison with that in low-dose diosgenin ($^{\&}P < 0.05$, $^{\&\&}P < 0.01$).

Table 2: Effects of toxicity on lymphocytes (mean±standard deviation, *n*=6)

Group	A value	Survival rate (%)
Blank	0.214±0.005	-
Control	0.399±0.012	100.0
3.125 μmol/L diosgenin	0.387±0.009	93.8*
6.25 μmol/L diosgenin	0.375±0.015	87.4*
12.5 μmol/L diosgenin	0.367±0.013	82.7*
25 μmol/L diosgenin	0.359±0.012	78.7*
50 μmol/L diosgenin	0.319±0.007	57.1*

*P<0.05 compared with the control group

Table 3: Inhibitory effects of diosgenin on lymphocytes proliferation (mean±standard deviation, *n*=6)

Group	A value	Inhibitory rate (%)
Blank	0.246±0.005	-
Control	0.813±0.064	0.0
3.125 μmol/L diosgenin	0.765±0.052	8.3*
6.25 μmol/L diosgenin	0.691±0.069	21.5*
12.5 μmol/L diosgenin	0.531±0.061	49.8*
25 μmol/L diosgenin	0.404±0.049	72.2*
50 μmol/L diosgenin	0.299±0.016	90.5*

*P<0.05 compared with the control group

Diosgenin decreased the expression of RORγt in the collagen-induced arthritis model

Next, the expression of RORγt was evaluated in the CIA model. The obvious increase of RORγt mRNA and protein levels in model groups were upregulated than those in the controls [$^{*}P < 0.05$, Figure 4a-c]. High-dose diosgenin significantly attenuated the expression of RORγt mRNA levels [$^{\#}P < 0.05$, Figure 4a]. All three doses of diosgenin decreased RORγt protein levels [$^{\#}P < 0.05$, Figure 4b and c].

DISCUSSION

RA leads to bone erosion and joint damage, resulting in dysfunction and even disability.[24] An imbalance of $CD4^{+}$ T-cell subsets is considered an important cause in the development of RA.[25,26] In this study, lymphocytes isolated from the CIA mouse model demonstrated an obvious increase in the ratio of Th17 cells in $CD3^{+}$ T cells, and upregulated the expression of IL-17, IL-6, and RORγt. Diosgenin treatment efficaciously inhibited the Th17 cells proportion in $CD3^{+}$ T cells and also suppressed the expression levels of IL-17, IL-6, and RORγt in isolated lymphocytes. This shows the therapeutic effects of diosgenin in CIA mice.

Collagen II is a crucial autoantigen in the pathogenesis of RA and is usually employed to induce arthritis in animals.[27] IL-2, a regulatory cytokine, derived from Th cells in the immune system. It drives the proliferation of T-cells, the cytolytic activity of natural-killer cells, and promotes the differentiation of Tregs cells.[28] In this study, chicken type II collagen and IL-2 were used to construct the CIA model, with obvious redness and swelling identified in the mouse paw. Simultaneously, 90% of the lymphocytes isolated from the lymph glands of the CIA model presented CD69+, indicating that the CIA model was successfully established.

Van Hamburg *et al.* revealed that Th17 cells from early RA induced the synovial fibroblasts to secret matrix metalloproteinases and proinflammatory cytokines.[29] Corvaisier *et al.* reported that IL-26 was overexpressed in RA synoviocytes, acting upstream of proinflammatory cascades increase the ratio of Th17 cells leading to the development of RA.[30] Aggravation of the inflammatory response and destruction of

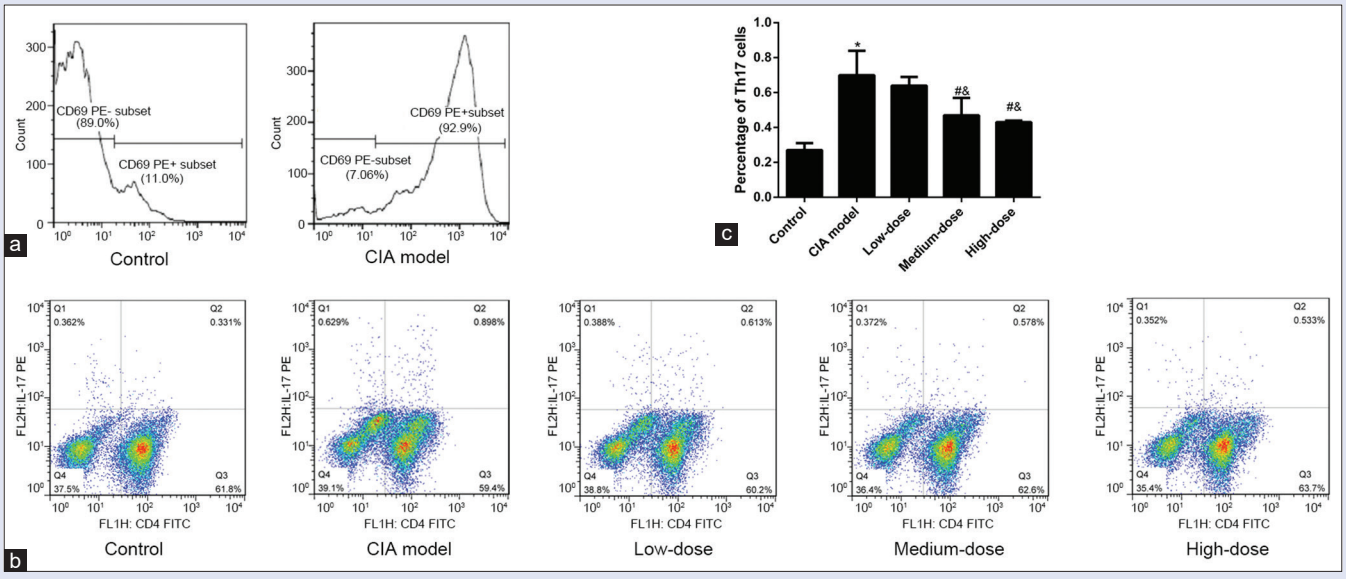

Figure 2: Effect of diosgenin extracted from *Dioscorea nipponica* Makino on T helper 17 cells. (a) Activation of T-cells in lymphocytes isolated from DBA/1J mice; (b) Ratio of T-helper 17 cells in isolated lymphocytes determined using flow cytometry; (c) Quantification of T helper 17 cell ratio in lymphocytes. $^{*}P < 0.05$ versus control group, $^{\#}P < 0.05$ versus collagen-induced arthritis model-dose group, and $^{\&}P < 0.05$ versus low-dose group

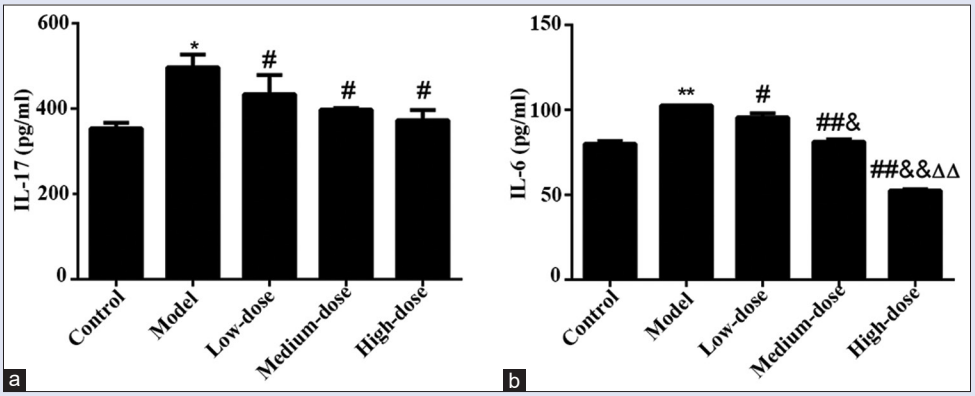

Figure 3: Expression of interleukin-17 and interleukin-6 after treating with diosgenin detected by enzyme-linked immunosorbent assay. (a) Interleukin-17; (b) Interleukin-6. **P* < 0.05 and ***P* < 0.01 versus control group, #*P* < 0.05 and ##*P* < 0.01 versus collagen-induced arthritis model group; &*P* < 0.05 and &&*P* < 0.01 versus low-dose group; ΔΔ*P* < 0.01 versus medium-dose group

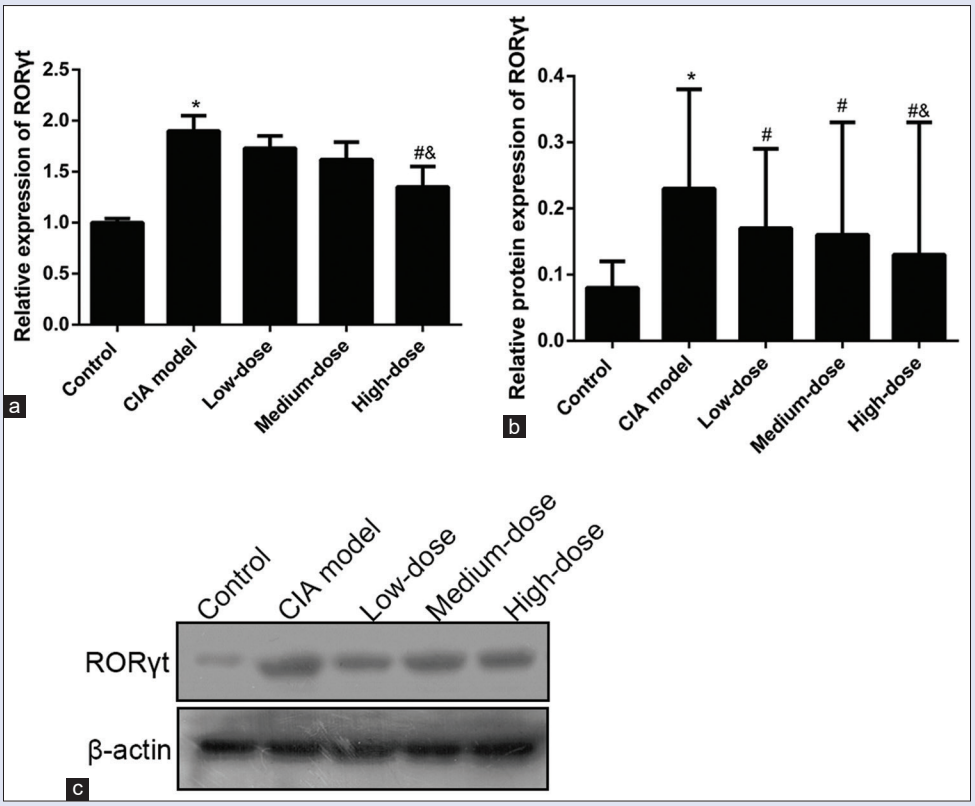

Figure 4: Expression of RORγt in lymphocytes after diosgenin treatment for 48 h. (a) mRNA expression of RORγt detected by quantitative real-time polymerase chain reaction; (b) Quantification of RORγt protein expression; (c) Expression of RORγt determined by western blotting. **P* < 0.05 versus control group, #*P* < 0.05 versus the collagen-induced arthritis model group, and &*P* < 0.05 versus low-dose group

collagen, bone, and cartilage tissues contributed to increased ratio of Th17 cells.[31-33] In this study, the ratio of Th17 cells in $CD3^+$ T-cells was obviously elevated in the model compared with that in the control. Notably, both medium- and high-dose diosgenin downregulated the ratio of Th17 cells compared with the model. These results confirm that the generation of Th17 cells may play a key role in the pathomechanism of CIA mice.

RORγt, a key transcription factor, is associated with the differentiation of Th17 cells and T-cell-mediated inflammation.[34,35] Patients with RA with better prognosis often present with reduced RORγt expression and Th17 cell ratio.[12] In an autoimmunity model, VTP-43742 selectively blocks RORγt, thereby suppressing the production of Th17 cells.[36] In this study, mRNA and protein expression levels of RORγt markedly raised in the model group, while significantly reversed by the diosgenin treatment. Based on these results, we inferred that diosgenin may inhibit the generation of Th17 cells by suppressing RORγt expression.

Th17 cells are induced by IL-6 and T-cell transforming growth factor-beta,[30,37] mainly secrete IL-17 and express RORγt.[38] Reportedly, the inhibition of IL-6 preferentially reduces the induction of the Th17 cells.[39] Furthermore, activated T cells with a high expression of RORγt produce large amounts of IL-17, whereas Th17 cells and IL-17A are

markedly suppressed in RORγt-knockout mice.[40] Diosgenin remarkably inhibits the Th1 and Th17 cell differentiation.[19] In CIA mice, dioscin contributes to the inhibition of Th17 cell differentiation and decreases the levels of IL-17, IL-6, and RORγt.[4] Dioscin also exerts effects in CIA mice by possibly impairing the differentiation of Th17 cells and cell-secreted cytokines.[1] Consistent with these results, in this study, diosgenin treatment downregulated the Th17 cell differentiation and the yields of IL-17, IL-6, and RORγt in CIA mice. This shows that the inhibitory effect of diosgenin on Th17 cell differentiation may contribute to the antiarthritic effect of diosgenin.

CONCLUSION

Diosgenin significantly attenuated the differentiation of Th17 cells in the CIA model, providing further evidence to explore the potential effects of diosgenin treatment in RA. However, the experiment was conducted *in vitro*, and additional studies are warranted to further explore the effectiveness of diosgenin in RA *in vivo*.

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Conflicts of interest

There are no conflicts of interest.

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