Effects of dioscin on T helper 17 and regulatory T-cell subsets in chicken collagen type II–induced arthritis mice

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Abstract
Background: This study was conducted to investigate the treatment efficacies and immunological mechanisms of action of dioscin in mice with chicken collagen type II–induced arthritis (CIA).

Methods: The CIA mice was randomly divided into the model group (M), dioscin group (D), and tripterygium group (T); a normal control group (C) was also included. Each group was orally administered with related drugs or an equal volume of solvent (group C) starting on the 21st day of primary immunity, after which the levels of T helper 17 cells (Th17), regulatory T cells (Tregs), and their related factors were detected on the 35th day.

Results: Compared to group C, group M exhibited significantly increased levels of interleukin 17 (IL-17) and IL-6 and decreased IL-27 (p < 0.05). Group D exhibited significantly decreased levels of IL-17 and IL-6 compared with group M (p < 0.05). Group M showed a significantly increased ratio of Th17 cells (p < 0.05), while dioscin significantly reduced this ratio (p < 0.05). Groups M and C showed no significant difference in the ratio of Tregs (p > 0.05) but dioscin significantly increased this ratio (p < 0.05). Group M significantly increased signal transducer and activator of transcription 3 (STAT3) and STAT5 compared with that in group C (p < 0.05), while the T and D groups showed significantly reduced levels of STAT3 and STAT5 (p < 0.05).

Conclusion: Dioscin may affect the differentiation of Th17 and Tregs and secretion of related factors by regulating CD4+ T cell subset-related signal transduction and the expression of transcription-activating factor STAT3 and STAT5, thus exerting useful immunoregulatory roles in CIA mice.

Keywords: CIA mice; Dioscin; Th17; Treg; STAT

1. INTRODUCTION
Rheumatoid arthritis (RA) is a common chronic autoimmune disease characterized by chronic erosive arthritis, lead articular cartilage, and bone destruction, which eventually lead to joint deformities and effects in other tissues, including the skin, blood vessels, heart, lungs, and muscles. The pathogenesis of RA is not clear, including heredity, environment, gender, and autoimmune abnormalities. Immune disorder is considered as the main mechanism of RA pathogenesis, particularly dysfunctions of the CD4+T cell subset. CD4+T cells are important in the body’s immune system. Currently, CD4+T cells are composed of four subsets of cells: Th1, Th2, Th17, and regulatory T cells (Tregs). CD4+T cell subsets differentiate from immature T cells (Tn), and Tn cells are differentiated into different T helper (Th) subgroups under different cytokine environmental conditions and signal transduction pathways. Studies have shown that different cytokines activate the JAK/STAT signal transduction pathway and further affect the differentiation of CD4+T cells. Interleukin (IL)-12 and interferon-γ induce Tn to differentiate into Th1 cells by activating STAT4 and STAT1 through up-regulation of the expression of transcription factor T-bet. IL-4 activates STAT6 to up-regulate transcription factor GATA3 expression and induce Tn to differentiate into Th2 cells. IL-6 can upregulate the expression of retinoid-related orphan receptor-γ-T through the synergistic actions of low concentrations of transforming growth factor (TGF)-β and STAT3 and induce Tn to differentiate into Tregs. Earlier studies suggested that Th1 plays a leading role in the occurrence of the tissue-specific autoimmune disease, RA. However, more recent studies showed that Th17 and Tregs play an important role in the development of RA.

A previous study showed that in collagen-induced arthritis (CIA) mice, diosgenin reduced paw swelling and arthritis index (AI). CIA model mice were orally administered with dioscin to investigate the efficacies of dioscin for treating RA, as well as to determine dioscin’s regulatory mechanisms for balancing Th17/Treg cellular immunity.
2. METHODS

2.1. Reagents and instruments

Dioscin (98%) was provided by Nanjing Spring & Autumn Biological Engineering Co., Ltd. (Nanjing, China); Th1/Th2/Th17/Th22 13plex reagents and antibodies for flow cytometry were provided by e-bioscience (California, USA); the BCA protein testing kit was provided by Thermo Fisher Scientific (Waltham, MA, USA); primary antibodies for STAT3 and STAT5 were provided by Abcam (Cambridge, UK). The FACS Calibur flow cytometer instruments were provided by BD Biosciences (Franklin Lakes, NJ, USA).

The positive control drug was tripterygium glycosides, which has been widely used to treat RA,12 glomerulonephritis, lupus erythematosus, and various autoimmune diseases and dermatitis. The strong immunomodulatory effects on CIA mice of the positive control drug have been demonstrated previously.13,14

2.2. Animals

Forty-eight male-specific pathogen-free DBA1/J mice, 7- to 8-weeks old (Shanghai Slaccas Experimental animal Co., Ltd., license number: SCXK (Hu) 2012-0002), were used in this study. All animals were housed in a specific pathogen-free animal facility 1 week before the experiment for adaptation.

2.3. Experimental grouping and modeling of CIA

After 1-week adaptive breeding, all DBA1/J mice were grouped using a random number table into the normal control group (C), model group (M), Dioscin group (D), and tripterygium group (T).

CIA is a classical experimental arthritis animal model established by Trentham in 1977. The pathological mechanism, histopathological and immunological changes were similar to human RA and was widely accepted internationally. The main mechanism of CIA includes T cell activation, cytokine secretion, and type II collagen antibody production. In this experiment, the “model group” was established according to Trentham’s modeling methods.14,15 First, Chicken type II collagen 2 mg/mL was mixed with an equal volume of Freund’s adjuvant and then emulsified via repeated aspiration in sterile tubes to form an emulsion of type II collagen. The mice in groups M, D, and T were intraperitoneally injected with 5% chloral hydrate (400 mg/kg) for anesthesia, and then subjected to multipoint intradermal injection of 0.1 mL of the emulsion into the tail vein under sterile conditions; the same dose was administered on day 21 for reinforcement. Group C was injected with the same volume of saline as described earlier.

The AI was used as a standard to measure the paw swelling of each mouse and determine the success of modeling. Limb joint swelling was scored as grades 0-IV: 0: no swelling; I: slight swelling at toe joint; II: swelling at toe and foot joints; III: swelling at paw and foot joints below the ankle; IV: swelling at all paw and foot joints including the ankle. AI value equals to the sum of all four limbs’ swelling scores (grade 0: 0 point, grade I: 1 point, grade II: 2 points, grade III: 3 points, grade IV: 4 points; a total of 16 points). An AI of ≥4 indicated the success of modeling. A higher AI value indicated more severe joint symptoms.16,17

2.4. Drug treatment

Sodium carboxymethyl cellulose (1%) was used to prepare a suspension of dioscin and tripterygium. Starting on day 21 of primary immunity, group D was orally administered with 100 mg/kg dioscin suspension, while group T was orally administered with the suspension of tripterygium tablets (17 μg/kg/d, calculated according to the instructions of Triptoline Tablets, and converted to the ratio of body surface area of human/mouse); groups C and M were orally administered with equal volume of solvent.

2.5. Sampling and index detection

The mice were decapitated on day 35 of primary immunity and then eyeball blood was collected under sterile conditions to prepare the serum, which was stored at −80°C to detect related cytokines, using a Mouse Th1/Th2/Th17/Th22 13plex detection kit. The mouse groin skin was then cut open under sterile conditions, the inguinal lymph nodes were isolated, and excess fat tissue was removed; the lymph nodes were then placed on 200-mesh steel mesh and gently ground to prepare a single-cell suspension of lymphocytes. After filtering to remove impurities, the prepared single-cell suspension of lymphocytes was seeded into 6-well plates and cultured at 37°C to remove mononuclear phagocytes. The cell concentration of each group was adjusted to 2 × 10⁶ cells/mL and 300 μL of the cell suspension was added to 96-well plates (2 wells for each mouse cell). Four additional wells were used to detect CD69 activity. To each well, we added 1.25 μL of phorbol-12-myristate-13-acetate (PMA)/Ionomycin mixture (LianheBio Co. Ltd., Taipei City, Taiwan, batch number: CS1001) for 1-hour culture at 37°C. Except for the CD69 detection well, the wells contained 1.25 μL of a brefeldin A (BFA)/Monensin mixture (LianheBio Co. Ltd., batch number: CS1002) and cultured at 5h at 37°C. To detect CD69, the isotopic control and test tubes were prepared and 100 μL of the cultured cell suspension was added to the flow tube, with the isotopic control tube containing 1.0 μL of Armenian Hamster IgG (e-bioscience Co., No: 12-4888), one tube containing 1.5 μL of CD69-PE (e-bioscience Co., No: 12-0691), and the remaining tubes containing 1 μL of CD3-FITC (e-bioscience Co., No: 11-4321), followed by 30-min culture in the dark at room temperature. After washing with PBS, the cells were resuspended and evaluated; if the CD69 ratio was >90%, the activation was defined as successful. The ratio of Th17 cells to CD3+ T cells was determined using intracellular antigen anti-mouse IL-17A PE, while the Tregs was determined by staining the surface antigens with anti-mouse CD4 FITC and anti-mouse CD25 PE.

The single-cell suspension was subjected to conventional lysis and extract and prepare protein samples. Sample concentrations were determined using a BCA protein concentration assay kit.

The lymphocytes collected from each group were mixed with pre-ice-cooled cell lysis buffer (RTPA:PMSF = 100:1) for lysis, centrifugation, extraction, and quantitation of total proteins according to the Assay Kit (Thermo Fisher Scientific, No: PG206438). An 8% separating gel and 5% stacking gel were prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis of 30 μg of samples for 2 h at a constant voltage of 120 V. Proteins were transferred to polyvinylidene fluoride membranes and blocked with 5% nonfat dry milk. The membrane was then incubated with 1:1000 p-STAT3 (Abcam) and p-STAT5 (Abcam) rabbit polyclonal antibodies overnight at 4°C, followed by 1:3000 horseradish peroxidase-labeled anti-lg antibody conjugate (Beyotime Biotechnology Co. Ltd., Shanghai, China, No. A0208) for 1 h. β-Actin was used as an internal reference. Conventional electrochemiluminescence staining was then performed, followed by imaging, scanning, and analysis.

2.6. Statistical analysis

In this study, SPSS22.0 software (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. All quantitative data were normally distributed and expressed as x ± s; intergroup comparison was conducted by one-way analysis of variance and post-hoc analysis was conducted by least significant difference (normal distribution) or Games-Howell (heterogeneity of variance), with p < 0.05 considered as a significant difference.

3. RESULTS

3.1. General observation

During the experiment, the mice in group C were stable throughout the experiment, while those in group M showed
reduced activities and eating, with back and tail roots forming multiple ulcers; their fur became darker and drier and the mice became apathetic and unresponsive. The mice in group D exhibited reduced systemic symptoms after medication, with improvements in their general mental conditions, increased appetite and activities, and shinier fur compared to mice in group M. Mice in group T exhibited better mental conditions than those in groups M and D, as well as better diet and shinier fur. The mice in group C showed no significant change in the footpad during the experiment and no foot and paw joint swelling; however, the mice in group M initially showed congestion and swelling of the rear feet, skin temperature increase, and footpad thickening, which gradually became involved in the rear knee joints and forelimbs and the joints could not bear weight; their body weights also decreased significantly. The conditions of foot and paw swelling in group D mice were reduced compared with those in group M. Group T showed the lowest paw swelling and footpad thickening (Fig. 1). AI in group C was maintained at 0 during the experiment, while that in group M reached 11.84 ± 3.48 on day 35, which was significantly different compared with that in group C (p < 0.05); AI in group D was significantly improved (5.77 ± 4.52) compared with that in group M. AI in group T showed the lowest value (4.88 ± 3.31), and the AI values in groups T and D exhibited significant differences compared with that in group M (p < 0.05) (Table 1).

### Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Weight, g</th>
<th>Paw swelling, mm</th>
<th>AI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group C</td>
<td>23.21 ± 1.22</td>
<td>1.41 ± 0.14</td>
<td>0 ± 0</td>
</tr>
<tr>
<td>Group M</td>
<td>19.82 ± 1.44</td>
<td>1.99 ± 0.13</td>
<td>11.84 ± 3.48</td>
</tr>
<tr>
<td>Group D</td>
<td>21.11 ± 1.52</td>
<td>1.80 ± 0.10</td>
<td>5.77 ± 4.52</td>
</tr>
<tr>
<td>Group T</td>
<td>20.84 ± 0.83</td>
<td>1.72 ± 0.18</td>
<td>4.88 ± 3.31</td>
</tr>
</tbody>
</table>

*p < 0.05 = compared with group C; **p < 0.05 = compared with group M; ***p < 0.05 = compared with group D.

3.2. Detection of cytokines by flow cytometry

Compared with group C, the levels of IL-17 and IL-6 in group M were significantly increased (p < 0.05), while IL-27 was significantly decreased (p < 0.05); IL-10 was increased, but the difference was not significant (p > 0.05). After oral therapy with dioscin, IL-17 and IL-6 were significantly decreased (p < 0.05) compared with group M; IL-10 and IL-27 showed an increasing tendency, but the difference was not significant compared with group M (p > 0.05; Fig. 2, Table 2).

### 3.3. Comparison of Th17 and treg cell ratio by flow cytometry

The ratio of CD69+ cells in the activation group was >90%, indicating that the activation was successful. The Th17 cell ratio in group M was significantly higher than that in group C (p < 0.05), but the Th17 cell ratios in groups D and T were significantly decreased compared with that in group C (p < 0.05); there was no significant difference in the Th17 cell ratio between groups D and T (p > 0.05) or between groups C and M (p > 0.05). The Treg cell ratio in group D was significantly higher than that in group M (p < 0.05) (Fig. 3, Table 3).

### 3.4. Detection of STAT3 and STAT5 expression by western blotting

Group M exhibited significantly increased expression of p-STAT3 compared with group C (p < 0.05); p-STAT3 was significantly decreased in groups D and T (p < 0.05). The expression in group D was more significantly decreased than that in group T (p < 0.05). Group M exhibited significantly increased expression...
of p-STAT5 than group C (p < 0.05) and that in group D was significantly increased compared with that in group M (p < 0.05), but the increase in group T was smaller (p < 0.05). Group M showed significantly increased expression of SOCS3 compared with group C (p < 0.05), but the difference between groups D and M was not significant (p > 0.05); the expression of SOCS3 in group T was significantly higher than that in groups M and D (p < 0.05); (Fig. 4, Table 4).

4. DISCUSSION

RA is a common chronic autoimmune disease characterized by synovitis that gradually becomes aggravated, eventually leading to joint deformities and a high disability rate. The pathogenesis of RA involves heredity, the environment, gender, and autoimmune abnormalities. Our earlier study revealed no differences in CD8+ T cells in CIA compared with that in control group. In contrast, CD4+ T cells are among the most important components of the immune system, which include Th1, Th2, Th17, and Tregs. Many studies have shown that RA is related to an imbalance in CD4+ T cells, particularly Th1 cells. Numerous studies have shown that Th17 and Tregs also play an important role in RA development.

Th17 cells play extremely important roles in the progression of RA by secreting IL-17, IL-21, or other cytokines; Th17 cells can promote chondrocytes and synovial fibroblasts to express matrix metalloproteinase and B receptor activator of nuclear factor receptor ligand, as well as promote destruction of the cartilage matrix. IL-17 is also a vascular growth regulator that can promote angiogenesis, resulting in pannus. CIA mice and RA patients have abnormally increased Th17 cells and serum IL-17 contents in vivo. Therefore, adjusting the ratio of Th17 cells and inhibiting the production of IL-17 can effectively relieve arthritic symptoms and reduce damage in the joints. Studies have shown that an IL-17 monoclonal antibody can significantly improve the symptoms of RA.

In this study, paw swelling was severe and the AI index was higher in the model group than in the control group, which is consistent with the severity of joint inflammation. After diosgenin

![Figure 2](image-url) Detection of cytokines by flow cytometry.

**Table 2**

Detection of cytokines by flow cytometry in different groups ($\bar{x} \pm s, n = 12$)

<table>
<thead>
<tr>
<th></th>
<th>IL-17, pg/mL</th>
<th>IL-6, pg/mL</th>
<th>IL-27, pg/mL</th>
<th>IL-10, pg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>131.78 ± 0.75</td>
<td>132.35 ± 2.44</td>
<td>388.49 ± 60.2</td>
<td>75.42 ± 27.3</td>
</tr>
<tr>
<td>M</td>
<td>133.77 ± 1.53</td>
<td>164.56 ± 33.6</td>
<td>268.21 ± 49.7</td>
<td>80.89 ± 40.7</td>
</tr>
<tr>
<td>D</td>
<td>131.85 ± 1.14</td>
<td>144.55 ± 14.36</td>
<td>299.98 ± 68.27</td>
<td>97.74 ± 45.21</td>
</tr>
<tr>
<td>T</td>
<td>131.98 ± 1.10</td>
<td>143.95 ± 6.29</td>
<td>349.67 ± 136.60</td>
<td>50.61 ± 36.47</td>
</tr>
</tbody>
</table>

*p < 0.05 = compared with group C; p < 0.05 = compared with group M.

The figure of paw of different groups.
administration, paw swelling relief and a decreased AI index was observed, indicating the positive effects of diosgenin on CIA. The serum levels of IL-17 and IL-6 in CIA mice were significantly increased and the Th17 cell ratio and p-STAT3 were significantly higher than in group C, which is consistent with previous studies. After treatment with dioscin, the serum levels of IL-17 and IL-6 were significantly decreased compared to that in group M and the Th17 cell ratio and p-STAT3 were significantly decreased, indicating that dioscin inhibits the expression of p-STAT3, thus inhibiting the differentiation of Th cells into Th17, reducing Th17-secreted IL-17, and producing therapeutic effects in CIA mice.

As a specific regulatory T cell subset, Tregs can prevent the activation of autoreactive T cells and inhibit the occurrence of autoimmunity. Tregs are crucial in maintaining immune homeostasis and preventing autoimmunity. In CIA mice, the ratio of Tregs to CD3+ cells was significantly lower compared to group C, indicating the reduced regulation of Tregs in CIA mice.

**Table 3**

Comparison of Th17 to CD3+ cells and Treg cell ratio to CD4+ cells by flow cytometry in different groups (X ± s, n = 12)

<table>
<thead>
<tr>
<th></th>
<th>Th17/CD3+</th>
<th>Treg/CD3+</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>1.00 ± 0.13</td>
<td>1.00 ± 0.09</td>
</tr>
<tr>
<td>M</td>
<td>3.76 ± 1.91a</td>
<td>1.16 ± 0.12</td>
</tr>
<tr>
<td>D</td>
<td>1.27 ± 0.12b</td>
<td>1.53 ± 0.44a</td>
</tr>
<tr>
<td>T</td>
<td>1.33 ± 0.14b</td>
<td>1.32 ± 0.08</td>
</tr>
</tbody>
</table>

*p < 0.05 = compared with group C; p < 0.05 = compared with group M; p < 0.05 = compared with group D.

**Table 4**

Relative protein expression of STAT3 and STAT5 in different groups (X ± s, n = 12)

<table>
<thead>
<tr>
<th></th>
<th>p-STAT3</th>
<th>p-STAT5</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>1.00 ± 0.12</td>
<td>1.00 ± 0.18</td>
</tr>
<tr>
<td>M</td>
<td>3.25 ± 0.60a</td>
<td>3.30 ± 0.55a</td>
</tr>
<tr>
<td>D</td>
<td>1.52 ± 0.43ab</td>
<td>6.37 ± 0.88ab</td>
</tr>
<tr>
<td>T</td>
<td>2.30 ± 0.10abc</td>
<td>4.38 ± 0.46abc</td>
</tr>
</tbody>
</table>

*p < 0.05 = compared with group C; p < 0.05 = compared with group M; p < 0.05 = compared with group D.

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**Fig. 3** Comparison of Th17 and Treg cell to the CD3+ cell ratio by flow cytometry.

**Fig. 4** Western blot: relative protein expressions of STAT3 and STAT5 in different groups.

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autoimmune diseases, thus playing important roles in maintaining self-tolerance. Studies have shown that introducing in vitro amplified Tregs in vivo or drugs that enhance the functions of Tregs can relieve disease symptoms. Tregs can secrete IL-10 and TGF-β, thus playing roles in immune regulation. IL-10 is an active suppressor of T cells and antigen-presenting cells and can inhibit the release of IL-1 and tumor necrosis factor α produced by monocytes, as well as can promote the generation of IL-1 receptor antagonist and soluble tumor necrosis factor-α receptor. In RA, IL-10 inhibits Th17 cell-mediated inflammatory responses, prompts the generation of Tregs, mitigates lymphocyte infiltration and activation of osteoclasts in synovial tissues, reduces swelling in the joints, and inhibits disease development, thus playing important immune suppression and immune roles. IL-27 belongs to the IL-12 superfamily, eases development, thus playing important immune suppression. In CIA mice, IL-27 treatment of CIA mouse has resulted in significant reduction of CIA score, indicating that IL-27 can prevent the differentiation of Th2 cells while promoting the secretion of Th1 cells and their cytokines. However, when Th1 cells are highly polarized, IL-27 can limit the strength and duration of the Th1-type immune response, thus protecting the body from being damaged by an excessive immune response; IL-27 can promote the differentiation of Tregs and inhibit the production of Th17, thus playing important roles in suppressing inflammation. No significant difference was found in IL-10 between groups M and C, indicating that a secretion disorder of IL-10 may prevent effective protection. IL-27 in group M was significantly reduced compared with that in group C, indicating that IL-27 is involved in the inflammatory response in the acute phase of CIA and immune regulation toward inflammation suppression fails. The mechanism may involve deficient regulation of the equilibrium in Th17/Tregs, as well as in their secreted cytokines IL-17 and IL-10. Additionally, Tregs and p-STAT5 signaling protein were significantly increased in group M, while Tregs were not increased significantly, indicating that Tregs prevent the suppression of autoimmune diseases. After treatment with dioscin, the p-STAT5 protein and Treg cell ratio were significantly increased, while the levels of IL-10 and IL-27 were not significantly increased, indicating that dioscin activates p-STAT5, thus inducing Tn to differentiate into Tregs, suppresses inflammation, and exerts protective functions in CIA mice. However, its regulatory roles on Tregs-secreted cytokines were not significant.

In conclusion, dioscin relieved the effects on paw and foot joint swelling in CIA mice possibly by inhibiting the differentiation of Th17 cells and inhibiting the generation of Th17 cell-secreted cytokines, accelerating the differentiation of Tregs to regulate the immune balance of Th17/Tregs.

There are some differences in Tregs between humans and mice, particularly the heterogeneity of Foxp3 Tregs. The CIA model was first established by Trentham, and the pathological mechanism, histopathology, and immunology were found to be similar to human RA.

Additional studies on human are needed, as animal experiments have confirmed the effectiveness of dioscin on RA, which is related to the increased Treg ratio.

ACKNOWLEDGMENTS

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Author Contributions: Enhong Xing and Yachun Guo are the corresponding author and contributed equally.

REFERENCES


