Protective effects of Jiayan Kangtai granules on autoimmune thyroiditis in a rat model by modulating Th17/Treg cell balance

Hou Yi, Wang Tieshan, Guo Xiangyu, Sun Wen, Guo Xuan, Wu Lili, Qin Lingling, Zhang Chengfei, Liu Tonghua

OBJECTIVE: To investigate the protective effects of Jiayan Kangtai (JYKT) granules, consisting of 9 Chinese herbs, in a rat model of autoimmune thyroiditis (AIT), and the possible underlying mechanism.

METHODS: Female Lewis rats (6-8 weeks) were randomly apportioned to 5 groups of 10, including a normal control. AIT was induced in the untreated AIT-model group, and rats treated subsequently with daily low, medium, or high dose JYKT granules. After 12 weeks, plasma levels of thyroid autoantibodies and morphological changes in the thyroid were detected by enzyme-linked immunosorbent assay and histological examination, respectively. The presence of interleukin (IL)-6, IL23p19, and IL-2 in thyroid tissue was assessed by immunohistochemical staining. The percentages of T helper (Th)17 cells and regulatory T cells (Tregs) in the peripheral blood were analyzed by flow cytometry. Relevant levels of cytokines and proteins were examined via bead-based multiplex flow cytometry and ELISA, respectively. Expressions of genes and proteins regulated by Th17 cells and Tregs were shown by real-time PCR and Western blot.

RESULTS: Compared to the control, AIT-model rats had higher plasma concentrations of thyroid autoantibodies. The high-dose JYKT rats showed significantly lower levels of thyroid autoantibodies compared with the AIT model group. Rats in the AIT-JYKT groups also had fewer thyroid lesions and less lymphocytic infiltration, a lower percentage of Th17 cells, and a higher percentage of Tregs, compared with the AIT-model. Rats given high-dose JYKT had a significantly lower Th17/Treg ratio compared with the AIT model. Differences in plasma cytokine concentrations and relevant gene and protein expressions in the spleens of JYKT-treated rats and the AIT group suggested an association between JYKT treatment and lower Th17 cell percentage and higher Treg activity.

Abstract

OBJECTIVE: To investigate the protective effects of Jiayan Kangtai (JYKT) granules, consisting of 9 Chinese herbs, in a rat model of autoimmune thyroiditis (AIT), and the possible underlying mechanism.
INTRODUCTION

Autoimmune thyroiditis (AIT), also known as Hashimoto’s disease, is an organ-specific autoimmune disease involving lymphocyte reactivity to the host thyroid. AIT is characterized by lymphocytic infiltration into the thyroid tissue, an enlarged thyroid gland, and the presence of serum autoantibodies against thyroglobulin and thyroid peroxidase. Additional clinical symptoms include pernicious anemia, diabetes, and other autoimmune diseases that compromise patients’ quality of life. The rate of AIT incidence is much higher in women than in men. The exact mechanism of AIT pathogenesis is not completely known. It is believed that genetic background, environmental factors, and endogenous factors contribute to the breakdown of immune tolerance, production of thyroid self-antigens, and subsequent activation of T lymphocytes. These interactions appear to trigger an inflow of lymphocytes into the thyroid gland and the production of anti-thyroid autoantibodies.

Helper T cells (Th cells), also known as CD4+ T cells, are a crucial component of immunological responses. Th1 and Th2 cells, differentiated from naive T cells, participate in the progression of AIT. In addition, multiple studies have indicated that Th1 and regulatory T cells (Tregs) also contribute to the pathogenesis and development of AIT. An imbalance between Th1 cells and Tregs has been observed, not only in mouse model of AIT but also in human samples. Specifically, an AIT mouse model indicated the induction of Tregs and reduced expression of a specific transcriptional factor, forkhead box P3 (FOXP3). This subsequently enhanced the Th17-specific transcription factor isoform: RAR-related orphan receptor gamma t (RORγt, encoded by the gene RAR-related orphan receptor C, or Rorc). Another independent study confirmed that peripheral blood mononuclear cells (PBMCs) from patients with AIT had a higher percentage of Th17 cells and higher mRNA expression of Rorc, accompanied by fewer Tregs and reduced FOXP3 levels compared with the healthy control group. A separate study showed that the AIT patients not only had significantly elevated intra-thyroid infiltrating Th17 cells, but also higher serum concentrations of interleukin (IL)-17 and IL-22 compared with control subjects. Moreover, the IL-17 levels in the thyroid were positively associated with local fibrosis, whereas the serum IL-17 concentration inversely correlated with patients’ residual thyroid function. Thus, all these studies indicate that regulating the balance between Th17 cells and Tregs may be an important therapeutic strategy in AIT.

Currently, no effective therapeutic strategies are available for AIT. The main methods, all of which are limited and risk severe side effects, include surgical treatment, thyroid hormone therapy, or immune-modulatory therapy. Thus, identification of a new effective agent for AIT treatment is urgently required. In this regard, we have focused our research attention on Traditional Chinese Medicine (TCM), which has a long history in the treatment of autoimmune diseases. Clinical evidence supports that TCM exerts beneficial effects in AIT treatment. For example, using an experimental rat model of AIT, it was observed that ginsenoside treatment led to lower levels of interferon (IFN)-γ in the peripheral blood. Ginsenoside also produced a biphasic effect on IL-4 secretion, where low and moderate doses promoted, but a high dose inhibited, its secretion. Another study evaluated the clinical efficacy of ruanjian xiaoying decoction on chronic lymphocytic thyroiditis. Similarly, another TCM recipe, xiaoyin, putatively functions by modulating the balance of Th1/Th2 cells.

The Jiayan Kangtai (JYKT) is a kind of traditional Chinese formula. We have observed that JYKT has demonstrable effects on AIT using a classic rat model of the disease. The work is the subject of a pending patent (Patent Application No. CN106421633A) filed by Beijing University of Chinese Medicine. However, the mechanisms that underlie the therapeutic effects of treatment with JYKT remain unclear.

This study was aimed to investigate the protective effects of JYKT granules in an AIT rat model, and the possible underlying mechanism was explored.

METHODS

Herbs and animals

The JYKT granules were obtained from Eastern Hospital of Beijing University of Chinese Medicine, China. It consists of 9 Chinese herbs: Chaihu (Radix Bupleuri Chinensis) 10 g, Yujin (Radix Curcumae Wenyujin) 20 g, Xiuwujia (Spica Prunellae Vulgaris) 30 g, Wumei (Prunus Mume) 15 g, Zhebeimu (Bulbus Fritillariae Thunbergii) 15 g, Xuanshen (Radix Scrophulariae) 10 g, Chuanxiong (Dipsacaceae nipponica) 10 g, Shancigu (Pseudo-bulbus Cuscutae) 6 g, and Huangqi (Radix Astragali Mongolic) 30 g.

Female specific pathogen-free Lewis rats (aged 6-8 week, 120-150 g) were purchased from Beijing Vital River Laboratory Animal Technology, China (Certificate of quality No. SCXK [jing] 2016-0006). The rats were housed in the Animal Facility of Beijing Institute for Drug Control, China at (24 ± 2) °C and ~ 40% hu-
midity, with free access to food and water. The Animal Care and Use Committee of local Institute for Drug Control approved all the animal procedures.

**AIT induction in rats and drug treatment**

AIT was induced in female specific pathogen-free Lewis rats, as described previously. Briefly, 100 mg of porcine thyroglobulin (T1126, Sigma, St. Louis, MO, USA) was dissolved in 50 mL of phosphate-buffered saline (PBS) and fully emulsified with complete Freund’s adjuvant (F5881, Sigma, St. Louis, MO, USA) in a 1:1 ratio. On days 1 and 7, 100 µg of the emulsion was injected subcutaneously into the back of the neck and body of the rat to induce immunization. Subsequently, on days 14, 21, 28, and 35, booster immunizations were administered by subcutaneous injection of 100 µg porcine thyroglobulin that was fully emulsified with incomplete Freund’s adjuvant (F5506, Sigma, St. Louis, MO, USA), prepared as described above.

The rats in the normal control group were given double-distilled drinking water, while rats for AIT induction were given 0.05% sodium iodide solution, from 1 week before the immune induction until the end of the experimental period. To verify the induction of AIT, at day 42, orbital venous blood was collected for testing the serum levels of thyroglobulin and thyroid peroxidase antibodies.

Fifty rats were randomly divided into the following 5 groups: untreated AIT model, AIT rats treated with low, medium, or high-dose JYKT, and the normal healthy control. The low, medium, and high JYKT group rats received intra-gastric JYKT at 0.708, 1.417, and 2.834 g/kg body weight, respectively, once daily for 12 weeks. The normal control and AIT group rats were given double-distilled water at the same volume of double-distilled water.

At the end of treatment, rats were anaeasthetized with 1% sodium pentobarbital, and 5 mL of blood was collected from the abdominal aorta into heparinized tubes. Plasma was isolated by centrifugation at 1000 × g for 10 min at 4 °C, and the supernatant was collected for measuring the thyroglobulin and thyroid peroxidase antibody levels and other cytokine assays. Erythrocyte lysis solution (130-094-183, Miltenyi Biotec, Bergisch Gladbach, Germany) was added to the pellet in the blood collection tube. After vortexing and incubating, the cells in the pellet were resuspended at a concentration of 5 × 10⁶ mononuclear cells/mL.

The bilateral thyroid glands were collected and fixed in 4% paraformaldehyde for pathological examination and immunohistochemical analysis. The spleen was collected and stored in liquid nitrogen for real-time reverse transcription-polymerase chain reaction (qRT-PCR) and western blot assay.

**Measurement of thyroid peroxidase and thyroglobulin antibodies by enzyme-linked immunosorbent assay (ELISA)**

The plasma concentrations of thyroid peroxidase antibody and thyroglobulin antibody were assessed by ELISA, using a thyroid peroxidase Ab ELISA kit (CSB-E11199R, Cusabio, Wuhan, China) and a thyroglobulin Ab ELISA kit (exp310551, EXPAND, Beijing, China), respectively, in accordance with the manufacturer’s instructions. Briefly, all samples were diluted 5-fold with PBS. Fifty microliters of the standard or sample was mixed with 50 µL of conjugate in each well of the plate and incubated for 1 h at 37 °C. Thereafter, the liquid was removed from each well, and the plate was washed. Fifty microliters of horseradish peroxidase-avidin solution was added to each well, and the plate was incubated for 30 min at 37 °C. After washing, 50 µL of substrate A and 50 µL of substrate B were added to each well and incubated for 15 min at 37 °C. Fifty microliters of stop solution were added to each well, and the optical density was determined using a microplate reader at a wavelength of 450 nm.

**Hematoxylin-eosin (HE) staining of thyroid glands**

The bilateral thyroid glands were collected from each rat, fixed in 4% paraformaldehyde, embedded in paraffin blocks, and cut into 4-µm sections for mounting onto microscope slides. The slides were stained with hematoxylin for 4 min, dehydrated, and then stained with eosin for another 3 min. Pathological examination of these HE-stained sections was performed independently by 2 physicians, under a light microscope (BX53, OLYMPUS, Tokyo, Japan) at 200 × magnification.

**Flow cytometric analysis of Th17 cells and Treg cells**

To assess the percentage of Treg cells, PBMCs were stained with anti-rat CD4 fluorescein isothiocyanate (FITC; 11-0040-82, eBioscience, San Diego, CA, USA) and anti-rat CD25 phycoerythrin (12-0390-82, eBioscience, San Diego, CA, USA) antibodies. For FOXP3 staining, the CD4- and CD25-gated PBMCs were incubated in a FOXP3 staining buffer set (00-5523-00, eBioscience, San Diego, CA, USA), for rupture of the membrane and fixation. Intracellular staining was conducted with anti-mouse/rat FOXP3 allophycocyanin (17-5773-82, eBioscience, San Diego, CA, USA) or anti-rat IgG2a isotype control allophycocyanin (17-4321-81, eBioscience, San Diego, CA, USA). Similarly, for Th17 cell assessment, the PBMCs were incubated at 37 °C for 5 h with stimulants (including phorbol ester, ionomycin, Golgi blocker, and Brefeldin A 1640 medium). They were then stained with anti-rat CD3 FITC (11-0030-82, eBioscience, San Diego, CA, USA) or anti-rat CD4 FITC antibodies. After membrane rupture and fixation, anti-mouse/rat IL17A phycoerythrin (12-7177-81, eBioscience, San Diego, CA, USA) or isotype control MS IgG1 KPA FTCL phycoerythrin (554680, BD, Franklin, NJ, USA) were used for intracellular staining. All PBMCs were incubated for
30 min at 37 °C with the specific antibody before analysis by flow cytometry (BD FACSCanto II, Franklin, NJ, USA).

Assessment of Th17- and Treg-related plasma cytokines using bead-based multiplex flow cytometry and ELISA

Aimplex Rat Single Factor Test Kits were used to test the plasma levels of IL6 (A311125), IL17A/CTLA8 (A311113), IL2 (A311117), and IL10 (A311109), as described previously. The plasma levels of transforming growth factor beta 1 (TGF-β1) were tested using a human multi-factor detection kit (B111206). All of these kits were purchased from Beijing Quantobio (Kuangbo) Biotechnology, China, and the levels were detected using flow cytometry (BD FACSCanto II, Franklin, NJ, USA).

IL23p19 levels were detected using an ELISA kit (CSB-EL011638RA, Cusabio Biotech, Wuhan, China) in accordance with the manufacturer’s instructions. Briefly, 100 µL of the standard or sample was added to each well of the plate and incubated for 2 h at 37 °C. After removing the liquid from each well, 100 µL of the biotin-labeled antibody was added and the plate was further incubated for 1 h at 37 °C. The plate was subsequently washed and 100 µL of horseradish peroxidase-avidin was added to each well for additional incubation for 1 h at 37 °C. After washing the plate, 90 µL of tetramethylbenzidine substrate was added to each well, and the plate was incubated for 15 min at 37 °C. Fifty microliters of stop solution was added to each well, and the optical density was determined at a wavelength of 450 nm using a microplate reader.

Immunohistochemistry-based analyses of IL6, IL23p19, and IL2 protein in thyroid tissues

For immunohistochemical staining, 4-µm-thick sections of thyroid tissue embedded in paraffin were cut, dewaxed, rehydrated, incubated with 0.01% Triton for 10 min, and then 3% H2O2 for another 10 min. The antigen was retrieved by heating the sliced tissue sections at 90 °C to 98 °C for 12 min in a microwave oven. The sections were blocked with goat serum for 30 min and then incubated serially with the following primary antibodies overnight at 4 °C: IL6 (ab192271, Abcam, Cambridge, UK) diluted at 1: 5000; IL23p19 (sc-50303, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1: 500; and IL2 (sc-7896, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 1: 400. The secondary antibody was added, and the sections were incubated at 37 °C for 20 min. Diaminobenzidine staining was conducted, and then counterstained with hematoxylin. Sections were mounted on slides and examined under a light microscope (BX53, OLYMPUS, Tokyo, Japan) at 400× magnification. In parallel, negative control sections were incubated with PBS instead of a primary antibody.

The presence and amounts of the stained proteins were quantified according to the degree of staining and the distribution of the dye, using Image-Pro Plus 6.0 software. Five microscopic fields were randomly selected from each section for image analysis.

Examination of Th17- and Treg-related gene expressions in the spleen via qRT-PCR

RNA was isolated from 100 mg of the total spleen tissue by homogenization in Trizol reagent (s12075, Coolsaver, Beijing, China). The RNA was reverse transcribed into cDNA using a GoScript reverse transcription system kit (a5001, Promega, Madison, WI, USA). The cDNA (20 ng/µL) was used for gene amplification via qRT-PCR under the following conditions: 95 °C for 30 s; and 40 cycles of 95 °C for 5 s and 60 °C for 30 s. The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (Gapdh) was used as the internal control. The upstream and downstream primers were designed using Primer Premier 5.0 software and synthesized by Shanghai Shenggong Bioengineering, China. IL17A: 5’-ACAGTGAAGGCAAGGTACT-3’, 5’-GCTCA-GAGTCCAGGTTGAAG-3’; IL10: 5’-CC-CAGAAATCAAGGAGCATT-3’, 5’-TCATTCTCTCAGGCTCTCACCAC-3’; Rorc: 5’-CATCTGACCCAAACCAGCCAAACCGA-3’, 5’-CGGGTGTGATAAGTTGTTAGGAACGG-3’; Foxp3: 5’-GAGTTTCTGGA-GACCTGCA-3’, 5’-CTGACCACCTTTTCTTGTGAACGGCA-3’; GAPDH: 5’-CAACTCCCTCAAGATTGTCAGCAA-3’, 5’-GAGTTTCTGGA-GACCTGCA-3’. The relative expressions of the target genes in each group were calculated using the \(2^{-\Delta\Delta C_t}\) method, where CT is the threshold cycle.

Examination of RORy and FOXP3 protein levels in the spleen via western blot

One hundred milligrams of spleen tissue was ground and lysed in lysis solution containing protease inhibitor, and then kept on ice for 30 min. During this period, the lysis solution was mixed several times. After centrifugation at 13 400 × g for 10 min, the supernatant was aspirated, and the protein concentration was determined. Twenty micrograms of protein samples were mixed with the sample buffer and boiled for 10 min. Protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to polyvinylidene fluoride (PVDF) membrane. After blocking with 5% nonfat dry milk for 1 h, the membrane was probed with primary antibody against β-actin (1: 1000, #4970, Cell Signaling Technology, Boston, MA, USA), RORy (1: 1000, ab78007, Abcam, Cambridge, UK), or FOXP3 (1: 1000, ab22510, Abcam, Cambridge, UK) overnight at 4 °C. The membrane was washed 3 × in tris-buffered saline and Polyorbate 20 (TBST), and incubated with horseradish peroxidase-secondary antibody at room temperature for 1 h. After washing, Su-
perSignal ultra-sensitive chemiluminescence (ECL) substrate (Thermo Scientific, Waltham, MA, USA) was applied on the membrane. The image was taken and analyzed using ImageJ software. The protein levels of RORγ and FOXP3 were normalized to the density of β-actin bands.

**Statistical analysis**
All data were analyzed using the statistical analysis software, SPSS 16.0 (SPSS Inc., Released 2007. SPSS for Windows, Version 16.0). Normally distributed continuous variables were expressed as mean ± standard error of mean, and tested by one-way analysis of variance with post-hoc multiple comparisons. Non-normally distributed or unequal variance data were tested using the rank sum test. A P value < 0.05 was considered statistically significant.

**RESULTS**

Fifty rats in 5 groups all completed the study. The successful induction of AIT in rats was confirmed by the significantly higher plasma levels of thyroid peroxidase and thyroglobulin antibodies at day 42, compared with the normal control group (data not shown). During the 12-week period of treatment, no toxicity reactions were observed. At the end of the 12 weeks, the mean body weights of each of the treatment groups and control were statistically similar (Table 1).

At the end of the 12-week treatment period, the rats in the high-dose JYKT group had significantly lower plasma levels of thyroid peroxidase and thyroglobulin antibodies compared with the untreated AIT-model rats (P < 0.01, Table 1). The levels of thyroid peroxidase and thyroglobulin antibodies in the low- and medium-dose JYKT treatment groups were progressively lower than that of the untreated model, but the differences were not significant.

**Morphology of the thyroid gland and lymphocyte infiltration shown by histopathology**
At the end of the 12-week treatment period, the bilateral thyroid glands of each rat were processed for HE staining (Figure 1). The thyroid follicles of the normal control rats were round- or oval-shaped, with inactive columnar epithelium (Figure 1A). The follicular cavity appeared to be filled with visible reddish colloid, and no lymphocyte infiltration was observed in the acinar tissue or its surrounding interstitial space. In contrast, the structures of the thyroid tissue sections from the untreated AIT-model group were compromised, with damaged and atrophied thyroid follicles (Figure 1B); the colloid was thin in the acinar cavity, and infiltrating lymphocytes were observed in the interstitial space. In the low, medium, and high dose JYKT treatment groups (Figures 1C-E), the thyroid lesions were fewer compared with the untreated AIT-model rats, and the overall structure showed relative integrity, despite some infiltration of lymphocytes in the interstitium.

### Table 1. Body weight and plasma concentrations of thyroid peroxidase and thyroglobulin antibodies of the 5 groups at the end of 12 weeks of treatment

<table>
<thead>
<tr>
<th>Group</th>
<th>Body weight (g)</th>
<th>TPO Ab (IU/mL)</th>
<th>Tg Ab (µg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>237.86±7.81</td>
<td>4.59±0.28</td>
<td>0.95±0.04</td>
</tr>
<tr>
<td>AIT-model</td>
<td>228.51±5.46</td>
<td>333.35±12.92</td>
<td>12.15±0.37</td>
</tr>
<tr>
<td>JYKT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>230.98±3.78</td>
<td>314.17±8.41</td>
<td>10.91±0.95</td>
</tr>
<tr>
<td>Medium</td>
<td>235.94±3.05</td>
<td>306.65±12.49</td>
<td>10.25±0.95</td>
</tr>
<tr>
<td>High</td>
<td>232.48±3.49</td>
<td>257.72±10.70</td>
<td>9.72±0.66</td>
</tr>
</tbody>
</table>

Notes: Ab: antibody; BW: body weight; Tg: thyroglobulin; TPO: thyroid peroxidase. The data are expressed as mean ± standard error of mean and were analyzed by rank sum test due to abnormal distribution; “P < 0.01 vs control, “P < 0.01 vs AIT (n = 10).
**Th17 cell and Treg cell results shown by flow cytometry**

To assess the percentages of Tregs and Th17 cells, PBMCs were processed with specific antibodies, stained, and analyzed via flow cytometry (Figure 2). Compared with the normal control rats, the percentage of Treg cells of the untreated AIT-model rats was significantly lower (Figures 2-2E and 2-3B), but the percentage of Th17 cells was significantly higher (Figure 2-1E and 2-3A). Therefore, the Th17/Treg ratio of the untreated AIT-model was higher than that of the control (Figures 2-3C).

The Th17 cell percentage and Th17/Treg ratio of the high-dose JYKT treatment groups were significantly lower than that of the untreated AIT-model rats, while the Treg cell percentage was significantly higher (Figures 2-3A, B, C).

The percentages of Th17 cells (Treg cells) in the low- and medium-dose JYKT treatment groups were progressively lower (higher) than that of the untreated model, but the differences did not reach significance. However, the Th17/Treg ratios of both the low- and medium-dose groups were significantly lower than that of the untreated model.

**Plasma concentrations of cytokines regulated by Th17 or Treg cells**

Compared with the normal control rats, the plasma concentrations of IL6, IL17A, TGF-β1, and IL23p19 of the untreated model rats were significantly higher (Figures 3A-D), while the concentrations of IL2 and IL10 were significantly lower (Figures 3E, F).

In the high-dose JYKT group, the concentrations of IL17A, TGF-β1, IL23p19 were significantly lower compared with the untreated AIT-model rats (Figure 3B-D), while the concentrations of IL2 and IL10 were significantly higher (Figures 3E, F).

In the low and medium dose JYKT groups, the plasma concentrations of IL10 were significantly higher than that of the untreated AIT-model group (Figure 3F). However, regarding IL17A, TGF-β1, IL23p19, and IL2, the trend in difference between the low- and medium-dose groups and the untreated model was the same as the high-dose group, without reaching statistical significance (Figures 3B-E).

**IL6, IL23p19, and IL2 protein levels in thyroid tissues revealed by immunohistochemistry**

Thyroid tissues were prepared for immunohistochemical staining to reveal the presence and levels of IL6, IL23p19, and IL2 proteins (Figure 4). The thyroid tissue samples from the normal control rats had low levels of IL6 and IL23p19 proteins, whereas the untreated AIT-model rats had significantly higher amounts of these proteins (P < 0.01). Thyroid tissue samples from the high-dose JYKT treatment group had significantly lower levels of IL6 and IL23p19 proteins compared with the untreated AIT-model group (P < 0.05 and P < 0.01, respectively).

IL2 protein levels were high in the normal control rats, but were significantly lower than that in the untreated AIT-model group (P < 0.01; Figure 4). Rats in the low and high dose JYKT treatment groups had significantly higher levels of IL2 protein compared with the untreated AIT-model (P < 0.05 and P < 0.01).

**Th17 cell- and Treg-related gene expressions (mRNA) in the spleen**

The normalized mRNA expressions of the genes Rorc, IL17A, IL10, and Foxp3 in spleen tissues were measured using qRT-PCR (Table 2). The mRNA expressions of IL17A and Rorc were significantly higher in the spleen of the untreated AIT-model rats compared with rats of the normal control group (P < 0.05 for IL17A; P < 0.01 for Rorc). In contrast, the mRNA levels of IL10 and Foxp3 were significantly lower in the AIT-model than in the normal control group (P < 0.01).

Compared with the untreated AIT-model, treatments with low-, medium-, or high-dose JYKT were associated with significantly lower levels of IL17A and Rorc mRNA, but significantly higher levels of IL10 and Foxp3 mRNA (Table 2).

**Effects associated with JYKT on the protein levels of RORγ and FOXP3 in the spleen of AIT rats**

Compared with the normal control group, the protein expression of RORγ in AIT group was significantly higher (P < 0.01) and the expression of FOXP3 was significantly lower (P < 0.01; Figure 5). After JYKT treatment, the high-dose group showed a lower protein level of RORγ (P < 0.01) and higher protein level of FOXP3 (P < 0.01) compared with the AIT group; the protein level of RORγ was also significantly lower in the middle-dose JYKT group (P < 0.05) compared with the AIT group.

**DISCUSSION**

This study investigated the effects of JYKT on AIT pathogenesis in a rat model and explored the underlying molecular mechanism. Female Lewis rats were selected to generate the AIT animal model because they are inbred, highly homozygous, and susceptible to autoimmune diseases. In addition, these rats show few individual differences and results have good reproducibility. After inducing AIT in these rats, we observed that there were significantly higher levels of plasma thyroid peroxidase and thyroglobulin antibodies compared with the normal control rats. The pathological analysis of the AIT rats revealed lymphocyte infiltration in the thyroid tissue accompanied by destruction and atrophy of thyroid follicles. This observation confirmed the successful induction of AIT and indicated that the model...
The present results showed that each of the JYKT granules against signs of AIT in the rat model. The present results showed that each of the 3 JYKT
cant in the rats of the high-dose group, while the trend in these plasma concentrations were statistically significant compared with the untreated AIT model. The differences in thyroid peroxidase and thyroglobulin antibodies, compared by ELISA. A: IL-6 levels were measured by ELISA. A: IL-6 levels were compared by rank sum test due to unequal variance; B-F: one-way analysis of variance was used. *P < 0.05; **P < 0.01 vs control; ***P < 0.05; ****P < 0.01 vs AIT (n = 4).

**Figure 3** Plasma levels of Th17 cell- and Treg-related cytokines
A: IL-6; B: IL-17A; C: TGF-β1; D: IL-23p19; E: IL-10. A, B, C, E, F measured by bead-based multiplex flow cytometry; D measured by ELISA. A: IL-6 levels were compared by rank sum test due to unequal variance; B-F: one-way analysis of variance was used. *P < 0.05; **P < 0.01 vs control; ***P < 0.05; ****P < 0.01 vs AIT (n = 4).

**Figure 4** IL6, IL23p19, and IL2 protein levels in thyroid tissues shown by immunohistochemical staining
A1-F1: IL6; A2-F2: IL23p19, A3-F3: IL2. A1, A2, A3: normal control; B1, B2, B3: AIT group; and low-(C1, C2, C3), medium-(D1, D2, D3), and high-dose (E1, E2, E3) JYKT treatment groups, respectively. F1, F2, F3: Negative control staining. G-I: bar graph comparison, the results of quantitative analysis of immunohistochemical staining using Image Pro-Plus 6.0 software. G: IL6; H: IL23p19, and I: IL2. G, H: comparisons of IL6 and IL23p19 were performed by rank sum test due to unequal variance. *P < 0.01 vs control; **P < 0.05; ***P < 0.01 vs AIT (n = 5).

doses were associated with lower levels of plasma thyroid peroxidase and thyroglobulin antibodies, compared with the untreated AIT model. The differences in these plasma concentrations were statistically significant in the rats of the high-dose group, while the trend in the low- and medium-dose groups were similar. This suggests that the concentration of the low and medium doses was insufficient to reduce the levels of autoantibodies. The parallel pathological examination of the thyroid tissues also showed fewer thyroid lesions and
less lymphocyte infiltration among the 3 JYKT groups compared with the thyroid pathology of the untreated AIT rats. This suggested that all 3 doses may have a therapeutic effect, although the greatest benefit was shown by the rats receiving the high dose. Although our data suggested that JYKT might have a protective effect in AIT rats, the underlying molecular mechanism remains unclear. Some studies have suggested that Th17 cells and Tregs contribute to the pathogenesis and development of AIT. Th17 cells and Tregs are usually differentiated from precursor cells and characterized by the secretion of cytokine subsets. One transcription factor, FOXP3, is highly and specifically expressed in Tregs and is believed to mediate Treg development in the thymus. Its expression and function reflects the level of Treg activity. In addition, TGF-β1 has been shown to stimulate the differentiation and maturation of Tregs, which subsequently secrete IL2, IL10, and other factors.

The biological function of Th17 cells is linked to cytokines such as IL17 (also known as IL17A), IL17F, IL21, and IL22. IL6 and TGF-β1 can promote the differentiation of Th17 cells by activating the phosphorylation of STAT3 (signal transducer and activator of transcription 3) and the positive feedback of IL21. In addition, the nuclear orphan receptor RORγt appears to coordinate diverse cytokine-induced signals and thus to control Th17 cell differentiation. Activated Th17 cells further promote levels of IL17A and initiate IL23-mediated inflammation.

Interestingly, Th17 cells and Tregs have opposing functions. It is believed that Th17 cells represent a pro-inflammatory subset, whereas Tregs have an inhibitory effect on Th17 cells. Thus, maintaining the immune balance between Th17 cells and Tregs is very important for host homeostasis under normal physiological conditions. Any alteration in the balance between these cell types can eventually lead to the pathogenesis of various autoimmune diseases, including AIT.

IL23 is a member of the IL12 family and has a shared p40 subunit with IL12 and a unique p19 subunit. These 2 subunits together perform biological functions, and therefore, we chose IL23p19 to measure, rather than IL23. Our present data showed that the percentage of Th17 cells and levels of the related cytokines, IL17A, IL6, IL23p19, and TGF-β1, were significantly higher in AIT model rats compared with the normal control rats. In contrast, the AIT model rats

Table 2 Expressions of Th17 cell- and Treg-related genes (mRNA) in the spleen

<table>
<thead>
<tr>
<th>Group</th>
<th>IL17a</th>
<th>Rorc</th>
<th>IL10</th>
<th>Foxp3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.03±0.14</td>
<td>1.01±0.06</td>
<td>1.01±0.04</td>
<td>1.01±0.08</td>
</tr>
<tr>
<td>AIT</td>
<td>2.99±0.17</td>
<td>1.61±0.13</td>
<td>0.54±0.03</td>
<td>0.53±0.03</td>
</tr>
<tr>
<td>JYKT Low</td>
<td>2.27±0.31</td>
<td>1.41±0.05</td>
<td>0.72±0.05</td>
<td>0.72±0.06</td>
</tr>
<tr>
<td>Medium</td>
<td>2.34±0.15</td>
<td>1.45±0.06</td>
<td>0.74±0.02</td>
<td>0.77±0.06</td>
</tr>
<tr>
<td>High</td>
<td>1.91±0.30</td>
<td>1.30±0.13</td>
<td>0.88±0.02</td>
<td>0.96±0.09</td>
</tr>
</tbody>
</table>

Notes: comparisons for IL17a between groups were analyzed by rank sum test due to unequal variance, while one-way analysis of variance was used to analyze differences in the expression of other genes. Normalized to Gapdh. The data are expressed as mean ± standard error of mean; *P < 0.05; **P < 0.01 vs control; ***P < 0.05; ****P < 0.01 vs AIT (n = 4).
had a lower percentage of Tregs and the related cytokines IL2 and IL10. Significant differences were also noted between the AIT model and control group with regard to mRNA levels of IL17A and Rorc, which were higher in the model, and IL10 and Foxp3, which were lower. The western blot results further confirmed higher protein levels of RORγt and lower protein levels of FOXP3 in the AIT group, compared with the normal control rats.

These observations indicated that an imbalance between Th17 cell and Treg immunomodulation may be involved in the AIT pathogenesis induced by an emulsion of thyroglobulin and Freund’s adjuvant, and are consistent with a previously published study. In the present study, the high-dose JYKT treatment was associated with a reduction in the percentage of Th17 cells, but a higher percentage of Tregs, and thus led to a significant reduction in the Th17/Treg ratio. Consistent with these changes, we also observed a corresponding alteration in gene and protein levels of related cytokines and specific transcriptional factors, thereby suggesting that the TCM JYKT may have a therapeutic effect in AIT via regulation of the imbalance between Th17 cell- and Treg-mediated immune functions. The low and medium doses of JYKT were not associated with significant effects on the percentages of Th17 cells and Tregs, but these groups did have a significantly lower Th17/Treg ratio. The higher levels of FOXP3 and IL17 suggested that JYKT may exert effects by promoting Treg differentiation, which will then inhibit Th17 cell function and further reduce the inflammatory response. Given that AIT is a chronic disease, and the course of treatment in the present study lasted for 12 weeks only, we speculate that low and medium doses of JYKT may lead to more significant protection with an extended treatment time.

Our study included only in vivo efficacy and mechanism experiments. Additional in vitro experiments may provide more insight into the detailed molecular mechanism of AIT pathogenesis. Moreover, given the multiple components of JYKT granules, the complete analysis of the individual ingredients of JYKT should be informative. Our further study will also conduct profile analyses of toxicity and safety associated with JYKT, which are necessary to determine its therapeutic utility. In summary, our study suggested that JYKT granules were associated with the reduction in thyroid peroxidase and thyroglobulin antibody levels in AIT-model rats, with a corresponding reduction in lymphocyte infiltration, and lessening of damage to thyroid tissue. In addition, the mechanism underpinning its action appears to involve correction of the Th17 cell and Treg immune imbalance in AIT and subsequent reduction in the inflammatory response.

ACKNOWLEDGMENTS

We thank Wu Lijuan, Dong Xiaoke, and Deng Li for technical assistance. We also thank Medjaden Bioscience Limited for English language assistance and scientific editing.

REFERENCES

16. Cui SL, Yu J, Shoujun L. Iodine intake increases ip-10 expression in the serum and thyroid of rats with experi-


