5,6,7,8-Tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidine derivative attenuates lupus nephritis with less effect to thymocyte development

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Abstract
Retinoic-acid-receptor-related orphan nuclear hormone receptor gamma t (RORγt), a critical transcriptional factor of Th17 cells, is a potential therapeutic target for Th17-mediated autoimmune diseases. In addition, RORγt is essential for thymocyte survival and lymph node development, and RORγt inhibition or deficiency causes abnormal thymocyte development, thymus lymphoma, and lymph node defect. Recent study demonstrated that specific regulation of Th17 differentiation related to the hinge region of RORγt. In this research, we investigated the effect of RORγt inhibitor, 5,6,7,8-tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidine derivative (TTP), in the therapy of lupus nephritis and its safety on thymocyte development. We demonstrated that TTP repressed the development of Th17 cells and ameliorated the autoimmune disease manifestation in the pristane-induced lupus nephritis mice model. The treatment of TTP in the mice did not interfere with thymocyte development, including total thymocyte number and proportion of CD4⁺CD8⁺ double-positive populations in the thymus, and had no substantial effects on the pathogenesis of thymoma. The TTP had a stronger affinity with full-length RORγt protein compared with the truncated RORγt LBD region via surface plasmon resonance, which indicated TTP binding to RORγt beyond LBD region. Molecular docking computation showed that the best binding pocket of TTP to RORγt is located in the hinge region of RORγt. In summary, as a RORγt inhibitor, TTP had a potential to develop the clinical medicine for treating Th17-mediated autoimmune diseases with low safety risk for thymocyte development.

Keywords RORγt · Th17 · Lupus nephritis · Thymocyte development

Introduction
Systemic lupus erythematosus (SLE) is a chronic autoimmunity driven by the production of multiple self-antibodies, such as anti-dsDNA antibodies and antinuclear antibodies (ANAs), which are canonical parameters and diagnostic criteria in clinic [1, 2]. All of these self-antibodies are important for the pathology of lupus nephritis (LN) which is the key lethal factor for SLE [2]. Th17 cells, a novel effect T cell subset producing IL-17A [3], have an important role in the pathogenesis of autoimmunity [3–6]. IL-17A, a pro-inflammatory cytokine [7], has a central role in the pathogenesis of LN [8, 9]. IL-17A abnormally elevates in multiple autoimmune mice model, such as rheumatoid arthritis (RA) [10] and LN model [11]. Recently, several drug treating psoriasis and rheumatoid arthritis via inhibiting the function of Th17 cells have been approved in the clinical application [12, 13].

RORγt, a member of retinoic-acid-receptor-related orphan nuclear hormone receptor (ROR) family [14], is
extensively expressed in the immune system [15, 16]. It is essential for the positive selection of thymocytes and CD4+CD8+ (double-positive, DP) thymocyte survival [17]. Previous study revealed that RORγt-deficient mice could disturb T cell development, increase apoptotic thymocytes, and alter the proportion of CD4/CD8 subpopulation in the thymus [18]. RORγt consists of three domains [19, 20]: a highly conserved DNA-binding domain (DBD) to response DNA binding, a conserved ligand-binding domain (LBD) with an AF2 domain to recruit SRC family of co-activators for stimulating gene expression [21], and a hinge domain (HD) linking a DBD with a LBD. Previous studies showed that RORγt inhibitors prevented the development of Th17-dependent autoimmunity, as well as interfered T cell development in the thymus [22, 23]. Recent studies indicated that some regions in the HD of RORγt are required for the suppression of Th17 differentiation but not for the development of the thymus and lymph node [24]. It suggested that the HD of RORγt was a target for specifically inhibiting Th17 function.

RORγt is a master transcriptional factor for the differentiation of Th17 cells [25] and affects the function of Th17 cells by inducing the expression of IL17A gene and IL17F gene [25]. RORγt-deficient T cells displayed a significant reduction of Th17 differentiation, and RORγt-deleted mice demonstrated more resistance to EAE due to impairing Th17 differentiation [25]. Many studies demonstrated that RORγt could be a potential therapeutic target for Th17-mediated autoimmune diseases [26–28]. More than two hundred small-molecular compounds have been patented, which could inhibit the activity of RORγt, block Th17 differentiation, and ameliorate the clinical manifestation of autoimmune disease [27, 29]. In previous studies, we found that tetraazacyclic compounds had a potentiality to inhibit RORγt transcriptional activity [30]. 5,6,7,8-Tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidine derivative (TTP) belongs to the tetraazacyclic compounds family and displays excellent inhibitory effect on RORγt activity, so it is chosen as the target compound for this study. The structure of TTP was described in the previous article [31] (Supplementary Fig. 1). In this study, we further reveal the function of TTP on the subset T cell differentiation. Furthermore, we also demonstrate that TTP attenuates the manifestation of lupus nephritis. We investigate the effect of TTP on thymic development and observe its effect on the incidence of T cell lymphoma. Surface plasmon resonance is employed to detect the affinity of TTP to RORγt protein, as well as reveal the binding pocket of TTP to RORγt via molecular docking computation. With these studies, we evaluate the potential of TTP as the lead compound for the therapy of Th17-mediated autoimmune diseases.

Materials and methods

Mice

Two-month-old C57B6/J female mice, 2-month-old BALB/C female mice, and 1-month-old BALB/C female mice were purchased from the National Resource Center for Mutant Mice of China (Nanjing, China). All of mice were bred and housed under specific pathogen-free conditions in Sun Yat-sen University Laboratory Animal Center (Guangzhou, China). C57B6/J female mice were used for T cell differentiation and thymocyte apoptosis in vitro, 2-month-old BALB/C female mice were used for pristane-induced mice model of lupus nephritis, and 1-month-old BALB/C female mice were used for studying the development of the thymus.

T cell differentiation in vitro

Naïve CD4+ T cells were purified from C57B6/J mice splenocytes by a MACS magnetic column with a CD4+ T cell negative enrichment kit according to the manufacturer’s protocol (eBioscience, USA). The suspension of 1 × 10⁶ cells/ml RPMI-1640 containing 100 U/ml penicillin, 100 mg/ml streptomycin, and 10% FBS were cultured in 12-well plates pre-coated with anti-CD3e antibody (5 μg/ml, eBioscience) and anti-CD28 antibody (2 μg/ml, eBioscience). The medium contained different cytokines or antibodies as following: mouse IL-6 (30 ng/mL, R&D Systems, Minneapolis, MN, USA), human TGF-β (5 ng/mL, R&D Systems), mouse IL-1β (20 ng/mL, R&D Systems), anti-mouse-IL-4 antibody (5 μg/ml, ebioscience), and anti-mouse-IFN-γ antibody (5 μg/ml, ebioscience) for Th17 differentiation; mouse IL-12 (20 ng/ml, PeproTech), mouse IL-2 (10 ng/ml, PeproTech), and 5 μg/ml anti-mouse IL-4 antibody for Th1 differentiation; and 10 ng/ml mouse IL-2, 10 ng/ml human TGF-β, 5 μg/ml anti-mouse IL-4 antibody, and 5 μg/ml anti-mouse IFN-γ antibody for Treg differentiation. One day after activation, the T cells were treated with TTP, for 2 days. At day 3, cells were incubated with 50 ng/ml PMA (Sigma-Aldrich), 1 μg/ml ionomycin (Sigma-Aldrich), and 1 μg/ml brefeldin A (BD Biosciences) for 6 h in a tissue culture incubator at 37 °C and then be collected for flow cytometry analysis.

Apoptosis assay

Thymocytes were freshly isolated from 2-month-old C56B6/J mice and cultured in RPMI 1640 medium
supplemented with 10% FBS, 100 U/ml penicillin–streptomycin, and 2 mM L-glutamine at 1 × 10⁶ cells/ml. Cells were treated with TTP at different doses and time course: annexin V-FITC (eBioscience, Cat. No. 11–8005–74) and PI staining (eBioscience, Cat. No. 00–6990–42) analysis for dead cells at different time points.

**Mice model with lupus nephritis induced by pristane**

Two-month-old BALB/C female mice were induced to lupus nephritis by intraperitoneal injection with 500 μL pristane (Sigma-Aldrich). Control mice were received an equal volume of saline at the same way injection (n = 6). After 4 months, model mice were treated with TTP and prednisone acetate. The grouping is as follows: (1) the model group treated with 25% ethanol and 75% cyclodextrin (n = 15); (2) the positive drug group treated with prednisone acetate (15 mg/kg, n = 13); and (3) the TTP-treated group treated with TTP (15 mg/kg, n = 16). All drugs were dissolved with 25% ethanol and 75% cyclodextrin, and each mouse was intragastric administration with 100 μL drugs twice a week for 2 months.

**Enzyme-linked immunosorbent assay (ELISA) for serum anti-dsDNA antibody detection**

Serums were collected at the following time points: 2-month-old mice (before pristane injecting), 6-month-old mice (4 months after pristane, i.e., before drug-treated), 7-month-old mice (1 month after drug-treated), and 8-month-old mice (i.e., 2 months after drug-treated; ending of experiment). Serum anti-dsDNA antibodies were detected by ELISA with the homemade ELISA kit and the protocol for detection as described previously.

**Renal pathology and immune complex deposition**

**Pathological analysis of the kidney**

Kidneys were soaked in 4% polyoxymethylene for 1 day, embedded in paraffin, cut at 5 μm thickness. Sections were stained with periodic acid–Schiff stain (PAS).

**Detection of renal immune complex deposition**

Kidney sections were stained with Alexa Fluor 488 goat anti-mouse IgM (μ chain) and Alexa Fluor 488 goat anti-mouse IgG (H + L). The antibodies were diluted at 1:200. The deposition of immune complex of IgM or IgG in glomeruli was measured via fluorescence intensity which randomly selected a total of 15–20 glomerulus per section and scored blindly on a scale of 0–3 (0, none; 1, weak; 2, moderate; and 3, strong).

**Thymic development of mice**

One-month-old BALB/C mice were treated with 25% ethanol and 75% cyclodextrin (vehicle group, n = 10) and TTP (TTP group, 15 mg/kg, n = 10) and healthy group (n = 8) without any treatment. Collecting thymus and spleen of mice to detect the proportion of cells, the development of thymus, and the apoptosis of thymocytes when the mice were two months old. The apoptosis of thymocytes were detected by In Situ Cell Death Detection Kit, TMR red (Roche, Cat. No. 12156792910). The positive cells in random five views were detected by a fluorescence microscope (Lump, Japan). The ratio of apoptotic cells was calculated as positive cells/total cells. The mice were sacrificed and collected cells from the thymus and spleen to detect the proportion of lymphocytes at the age of three months. The T cell lymphoma development in the thymus was detected by H&E staining.

**Flow cytometry**

The flow cytometry analysis differentiation of T cell by following fluorescent antibodies was as follows: eFlour 450 anti-mouse CD4 (eBioscience, Cat. No. 48-0042-82), PE anti-mouse TCRβ (eBioscience, Cat. No. 12-5961-82), APC anti-mouse IL-17A (eBioscience Cat. No. 17-7177-87), PE anti-mouse IFN-γ (eBioscience Cat. No. 12-7311-82), and Alexa flour 680 anti-mouse Foxp3 (BD, Cat. No. 560401). The flow cytometry analysis proportion of cells in the spleen or thymus by following fluorescent antibodies was as follows: PECy7 anti-mouse CD4 (eBioscience, Cat. No. 25-0042-82), PE anti-mouse TCRβ (eBioscience, Cat. No. 12-5961-82), APC anti-mouse CD8e (eBioscience Cat. No. 17-0081-83), and FITC anti-mouse B220 (eBioscience, Cat. No. 11-0452-82). Data were analyzed by FlowJo software (FlowJo VX, BD).

**RNA isolation and quantitative real-time PCR**

Total RNA from the spleen was extracted using Trizol reagent (Invitrogen). Additionally, 1 μg RNA was reverse transcribed into cDNA using the PrimeScript RT Reagent Kit (Takara Bio, Japan). The associated gene expression was determined via the quantitative real-time PCR reaction (Takara Bio, Japan). The expression was calculated by the 2-ΔΔCt method normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

The primers were used in RT-qPCR as follows:
The recombinant plasmids of full-length RORγt and RORγt ligand-binding domain were transformed into *Escherichia coli* BL21 and used isopropyl-β-D-1-thiogalactopyranoside (IPTG, Sangon Biotech, Cat No. A600168-0005) to induce protein expression. The cells were lysed in HEPES buffer (20 mM HEPES pH 7.9, 1.5 mM MgCl2, 420 mM NaCl, 0.2 mM EDTA, 0.5 mM dithiothreitol (DTT), 25% glycerol) and ultrasonication. The proteins were purified with HisPur Cobalt Resin (Thermo scientific, Cat No. 89965). Full-length RORγt protein and RORγt LBD protein were immobilized on a CM5 Sensor Chip (carboxymethylated dextran covalently attached to a gold surface) with an amine coupling kit from GE Healthcare. The RORγt proteins were pre-incubated with different concentrations of TTP (60, 30, 15, 7.5, 3.75, and 1.875 μM) in a PBS buffer (6 mM) with 1% DMSO. The signals were recorded with a BiacoreT100 instrument with the standard protocol (Biacore T100, Sweden GE).

### Molecular docking

The binding between TTP with RORγt was detected via the molecular docking. The structure of full-length human RORγt was built with I-TASSER (idS428560). The 3D structure of TTP was prepared with MMFF 94 energy minimization by LigandScout (4.1) to the docking procedure. Then the protein structure was selected for docking, and the TTP was inserted. The docking score was obtained by AutoDock Vina 1.1.

### SPR

The difference between splenocytes from C57B6/J and BALB/c in Th17 cell differentiation in vitro was detected, which was no difference between them (Supplementary Fig. 2). Therefore, we chose C57B6/J for T helper cell differentiation in vitro in this study, and the purity of naïve CD4+ T cell was always over 95% via flow cytometry (Supplementary Fig. 3). We had discovered that TTP was a RORγt inhibitor in the previous report [31]. In this study, we compared the effect of TTP on different subset T cell differentiation in vitro (Fig. 1). Similar to the previous study, TTP inhibited Th17 differentiation (Fig. 1a, b). Compared with DMSO, TTP could significantly inhibit the expression of IL-17A and IL-17F (Fig. 1b), rather than RORγt (Supplementary Fig. 4a). TTP also inhibited Th1 differentiation with the reduction of IFN-γ secretion and the expression of Tbx21 (Fig. 1c, d). We observed that TTP had no effect on the Treg differentiation with the same expression level of Foxp3 and helios compared with DMSO (Fig. 1e, f).

### Ameliorating the clinical manifestation in the lupus nephritis mice model

**Reducing the serum dsDNA level in the lupus nephritis mice**

In order to evaluate the therapeutic potential of TTP on the Th17-related autoimmune diseases, we investigated the effect of TTP on the lupus nephritis mice induced by pristane. In this study, we induced a mouse model of lupus nephritis by injecting pristane into 2-month-old mice. The 6-month-old mice (4 months after injecting pristane) were treated with TTP twice per week. The serum anti-dsDNA antibody levels were examined at the following time points: 2 months of age (before pristane injection), 6 months (starting point of TTP treatment), 7 months (1 month after TTP treatment), and 8 months (2 months after TTP treatment). The results displayed that the serum anti-dsDNA antibody levels dramatically increased in the model group at 7 and 8 months compared with the control group (Fig. 2a).

### Results

**TTP specifically inhibits Th17 cell differentiation in vitro**

The statistical analysis

Prism software (GraphPad 7.0) was used for all statistical analyses. The statistical significance between groups was determined by one-way analysis of variance followed by Bonferroni’s test and Student’s t-test. A *p*-value of less than 0.05 was considered statistically significant. Data were expressed as mean ± SEM.

Target gene | Forward sequence | Reverse sequence |
|------------|------------------|------------------|
| GAPDH      | TGGTGAAGGTCG     | CCACGTAGTTGA     |
|            | GTGGGAC         | GGTCATAGGAAGG    |
| RORγt      | AAACCTGACAGC    | TGTAATGTTGCC     |
|            | ATCTGGAGA       | TACTCTCAGCA      |
| IL17A      | TTAAATCCCTTT    | CTTCCCCCTCCG     |
|            | GGCCGAAAAA      | ATTAGCAC         |
| IL17F      | GAGGATAAAGCT    | GAGTTCATGGTG     |
|            | GTGAGAGTTG      | CTGTCTTCC        |
| Tbx21      | AACACACACGTC    | CGTATCAACAGA     |
|            | TTATCTTCCA      | TGGCTACATGGG     |
| IFNγ       | GCCAGGCGACAG    | TCGGTAGGCCCT     |
|            | TCATTGAGA       | GATTGCTTT        |
| Foxp3      | GGCCTCTTCCCA    | GCTGATCATGGC     |
|            | GGACAGA         | TGGGTGTG         |
| Helios     | GACGCTGAGGGA    | CTCCCTCGCCTT     |
|            | TGAGATCAG       | GAAGTGC          |
Nevertheless, the serum anti-dsDNA antibody levels in the TTP-treated group were dramatically decreased compared with the model group, which is similar to the positive treatment group with prednisone acetate (Fig. 2a, b).

**TTP could alleviate renal damage in mice with lupus nephritis**

We next assessed pathological changes of the kidney via PAS staining; the results showed the larger volume of the glomerulus and enhanced lymphocyte infiltration and capillary hyperplasia in the mice with lupus nephritis compared with the control group (Fig. 2c, middle). It was ameliorated after TTP-treated (Fig. 2c, right) and clinical histopathological scores were significantly decreased (Fig. 2d). Moreover, we observed that the increment of anti-IgM antibody deposited in mouse model was reduced after TTP-treated through immunofluorescence analysis (Fig. 2e, f). The deposition of anti-IgG antibody in the kidney showed a resemble tendency with IgM (Fig. 2g, h). It indicated that TTP could alleviate renal damage with reducing the accumulation of immune complex in the lupus nephritis mice.

**TTP slightly inhibits Th17 cells in the spleen**

To further determine the effect of TTP on the Th17 function in the lupus nephritis mice, we detected the Th17 cells (Fig. 3a) and Th1 cells (Fig. 3b) in the spleen. These cells were slightly reduced under the treatment of TTP. Th17-related signature gene, including *IL17A* (Fig. 3c) and *IL17F* (Fig. 3d), were all slightly reduced in the presence of TTP. However, TTP did not affect the expression of RORγt in the spleen (Supplementary Fig. 4b).

**No interference with thymic development of mice**

Previous studies indicated that RORγt regulated thymocyte survival and the development of thymus. We wondered whether TTP could interfere with the development of the thymus. To clarify this point, we treated 1-month-old mice with TTP for 1 month. The results showed that the total cell numbers in the thymus had no significant difference with the treatment of TTP (Fig. 4a), as well as the population of different thymocytes, including CD4+CD8+ double-positive (DP), CD4+ single positive, CD8+ single positive,
**Fig. 2** TTP could significantly alleviate the incidence of mice with lupus nephritis. The anti-dsDNA antibody levels in each group were collected on 2 months old (pristane injection), 6 months old (4 months after pristane injection), 7 months old (1 month after TTP treatment, the endpoint of experiment), and 8 months old (2 months after TTP treatment, the endpoint of the experiment), and the antibody levels were detected by ELISA (a). The anti-dsDNA antibody levels in the 8-month-old mouse were shown (b). PAS-stained detected the volume of glomeruli and infiltration of lymphocytes (e), and clinical histopathological scores were shown according to PAS-stained (d). The deposition of anti-IgM antibody (e and f) and anti-IgG (g and h) antibody in the kidney were shown via immunofluorescence analysis. Additionally, 15–20 glomeruli were examined; the average scores and fluorescence intensity were obtained. Control group (n=6); model group (n=14); positive drugs, prednisone acetate (PA) (n=15); TTP-treated group (n=12). Scale bar were 100 μm (e), 50 μm (e), and 100 μm (g). Data were presented as mean±SEM. * p<0.05; ** p<0.01; *** p<0.001

and CD4−CD8− double-negative thymocytes (DN), were no difference among the TTP-treated group, healthy group, and vehicle group (Fig. 4b, c).

We also investigated whether TTP had an impact on the other peripheral immune organs. The frequency of lymphocytes in the spleen, as well as the proportion of T cell and B cell, had no significant difference between TTP-treated group and healthy group or vehicle-treated group (Fig. 4d, e). The proportion of CD4+ T cell and CD8+ T cell were also unaffected under treatment of TTP for 1 month (Fig. 4f, g).

**No effect on the thymocyte apoptosis and thymic lymphoma**

RORγt is a crucial transcriptional factor for preventing thymocyte apoptosis, and the defect of thymocytes would accelerate spontaneous apoptosis. In this study, we explored whether TTP had an influence on thymocyte apoptosis. The results showed that TTP only slightly increased thymocyte apoptosis under high concentrations in vitro (Fig. 5a and b and Supplementary Fig. 5). We also investigated the effect of TTP in the thymus with TUNEL assay to detect the apoptotic thymocytes in vivo. TTP slightly increased the proportion of apoptotic thymocytes compared with vehicle or healthy groups (less than 1%) (Fig. 5c, d). The apoptotic frequency of thymocytes was 7.4%, 7.8%, and 8.3% in healthy, vehicle, and TTP-treated groups, respectively (Fig. 5d).

Nevertheless, the lymphoma was not produced in the thymus of the mice with TTP-treated for 2 months (Fig. 5e). The thymus had a normal ratio of cortical to medullary (Fig. 5e, top and middle) and did not present larger thymocytes (Fig. 5e, bottom) following 2-month treatment with TTP. On the other hand, numerical and flow cytometric analysis revealed no difference in the numbers of total thymocytes and the frequency of thymocyte population (data not shown). These results indicated that TTP had no obvious side effects on the thymocyte development and thymoma pathogenesis.

**Interaction with hinge domain of RORγt**

We studied the binding affinity of TTP with RORγt protein via SPR. The results demonstrated that TTP had a stronger binding affinity with full-length RORγt protein compared to RORγt LBD protein (Fig. 6a, b), with the KD that was 44.1 μM and 99.4 μM, respectively. With molecular docking computation, the best binding pocket for TTP with RORγt protein is located at the amino acids in the hinge region, in which TTP could form electrostatic interaction with Gln223 and hydrophobic interaction with Leu244 of RORγt protein (Fig. 6c). These results suggested that TTP might interact with the hinge domain to regulate RORγt transcriptional activity.

**Discussion**

The CD4+ T cell subsets contribute to autoimmune disease pathogenesis, including Th1, Th17, and Treg cells. The balance between Treg cells and Th17 cells is crucially involved in autoimmune diseases. In this study, we did not observe the effect of TTP on Treg cells, which indicated that Treg were not the target of TTP. Some autoimmune disease etiologies also attribute to Th1 cells, including multiple sclerosis, RA, and SLE [32]. In this study, TTP suppressed Th1 differentiation in vitro and decreased the numbers of CD4+IFN-γ+ T cells in the LN mouse model, which indicated that Th1 cells could as the alternative target of TTP and coordinate with Th17 cells to alleviate LN pathogenesis. However, more efforts need to conduct for revealing the mechanism of TTP regulating Th1 function and the synergy with Th17 cells.

Lupus nephritis (LN) is a major cause of morbidity and mortality in SLE patients [33, 34], which has many representative clinical symptoms, including the increase of anti-dsDNA antibody level in serum, the infiltration of lymphocytes, and the deposition of immune complex in the kidney. The IgM and IgG complement-fixing antibodies to dsDNA deposit in the kidney relating to the risk of glomerulonephritis [35]. So far, the immunosuppressive agents for therapy of LN have had severe side effects because these agents work on multiple immune cells. Increasing evidences indicate the critical role of IL-17A and Th17 cells in SLE and other human autoimmune diseases [3–6], and they play an essential role in the pathogenesis of lupus nephritis [8, 9, 36]. In this report, we observed that the levels of anti-dsDNA antibody in serum were significantly
decreased after treating with TTP for 2 months, and the deposition of IgM or IgG immune complex in the kidney was markedly decreased with TTP treatment. These demonstrated that TTP could ameliorate the clinical manifestation of lupus nephritis in the mice, indicating its potential application in human SLE therapy.

RORγt, a central transcriptional factor in Th17 differentiation, serve as a potential therapeutic target for Th17-derived autoimmune inflammation[37, 38]. RORγt are also highly expressed in DP thymocyte subpopulation to enhance the survival of the DP thymocytes [39, 40]. Rorc-deficient (Rorc−/−) thymocytes would massively induce apoptosis, alter CD4/CD8 subpopulation, and lead to the emergence of lymphoma in the thymus [17, 18, 38, 41, 42]. Many studies showed that small-molecule agents, such as SR1001, digoxin, and ML209, as RORγt inhibitors, could inhibit the Th17 function [22, 27, 28, 43] with other side effects in the thymus, which included DP thymocyte apoptosis, downregulation of anti-apoptotic genes, and the induction of thymic lymphoma [23, 44]. In this study, we found that TTP disturbed the functions of Th17 cells, while it did not affect the numbers and frequency of CD4+CD8+ DP thymocyte population, and the total number of thymocytes had no significant difference with the control group. Furthermore, the subpopulation of T lymphocytes in the spleen had no difference in treating with TTP for 1 month and 2 months (data not shown). We also observed no significant increase of the large thymocytes in the mice treated with TTP for 2 months, and the deposition of IgM or IgG immune complex in the kidney was markedly decreased with TTP treatment. These demonstrated that TTP could ameliorate the clinical manifestation of lupus nephritis in the mice, indicating its potential application in human SLE therapy.

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with TTP, indicating no obviously pathological change in the thymus. The proportion of the DNA-damaged cells in the thymus was slightly increased after TTP treatment by TUNAL assay. These results indicated low safety risk of TTP on thymocyte development.

RORγt consists of three domains [19, 20]: a DBD to respond DNA binding, a conserved LBD with a AF2 domain to recruit co-activators stimulating gene expression [21], and a HD linking a DBD with a LBD. Previous study indicated that mutation of AF2 of RORγt interfered with the ability to activate target gene and the recruitment of co-activators [45]. However, disrupting the functions of RORγt LBD and DBD inhibited the IL-17A-mediated autoimmune inflammation, as well as the thymocyte

![Image](54x295 to 541x733)

**Fig. 4** The thymic development with treatment of TTP in the mice. The spleen and thymus were collected from 2-month-old BALB/C mice which were treated with TTP or vehicle for 1 month. The numbers of CD4⁺ T cell, CD8⁺ T cell, and total cell in the thymus (a). The frequency of CD4⁺ T cell and CD8⁺ T cell in the thymus, the representative flow analysis result (b), and the statistical result (c). The proportion of T and B cells in the spleen (d, e). The proportion of CD4⁺ T cell and CD8⁺ T cell in the spleen (f, g). The representative flow cytometric analysis was gated on CD3⁺B220⁺. Results were expressed at the mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001.
Previous study showed that two-amino-acid mutation in the HD of RORγt suppressed the Th17 function but not disrupted thymocyte survival [23]. HD region probably plays a crucial role in the discrimination of RORγt function between Th17 differentiation and thymocyte development. In this study, we demonstrated that TTP had a strong affinity with full-length RORγt rather than the RORγt LBD region, indicating that TTP might bind to HD of RORγt. Molecular docking computational analysis also demonstrated that the best pocket for TTP binding to RORγt located in the HD region. Therefore, the HD of RORγt may be the target region of TTP. It still remains to reveal the mechanism of TTP regulating RORγt function by interacting with the HD region.

![Fig. 5 Analysis of the thymocyte apoptosis and thymic alteration in the mice with treatment of TTP.](image)

The means of TUNEL+ cells were 7.36%, 7.79%, and 8.32% in the healthy group, vehicle-treated group, and TTP-treated group, respectively. Scale bar = 250 μm (e). N=5. The thymuses were collected from 3-month-old BALB/C mice which were treated with TTP or vehicle from 1 to 3 months old (e). H&E stained sections of the thymus from healthy (e, left), vehicle-treated (e, middle), and TTP-treated (e, right). The arrow points to the boundary between the cortex and the medulla in the thymus. The scale bars were 400 μm (e, top), 200 μm (e, middle), and 100 μm (e, bottom), N=6–7. Results were expressed at the mean±SEM. * p<0.05; ** p<0.01; *** p<0.001.
Conclusions

In summary, this study revealed that TTP repressed the differentiation of Th17 cells and ameliorated the clinical manifestation in the lupus nephritis mice model. Furthermore, TTP had no substantial effects on thymocyte development and the incidence of thymoma. These results indicated that TTP, a RORγt inhibitor, had a potent effect and low safety risk to develop the drug for treating Th17-mediated autoimmune diseases.

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Author contributions H.Z and B.C designed the study. W.F, Z.X, C.H, T.X, H.X, Y.B, and B.C performed the experiments and collected and analyzed the data. L.Z provided technical support on the mouse model. W.F, B.C, and H.Z drafted the manuscript. All authors read and approved the final manuscript.

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Data availability The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics declarations All animal experiments were approved and supervised by the Ethics Committee of ZSSOM on the Laboratory Animal Care (No. 2017–273) and were performed according to the guidelines of the Institute for Laboratory Animal Research of Sun Yat-sen University Laboratory Animal Center (Guangzhou, China).

Conflict of interest The authors declare no competing interests.

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