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

Fengjiao Wei
Institute of Human Virology, Department of Biochemistry and molecular biology, Zhongshan School of Medicine, Sun Yat-sen University

Xiaoqing Zhou
Institute of Human Virology, Department of Biochemistry and molecular biology, Zhongshan School of Medicine, Sun Yat-sen University

Huanpeng Chen
Institute of Human Virology, Department of Biochemistry and molecular biology, Zhongshan School of Medicine, Sun Yat-sen University

Xuyan Tian
Institute of Human Virology, Department of Biochemistry and molecular biology, Zhongshan School of Medicine, Sun Yat-sen University

Zhonghua Liu
Animal Experiment Center, South China Agricultural University

Bolan Yu
Key Laboratory for Major Obstetric Diseases of Guangdong Province, Third Affiliated Hospital of Guangzhou Medical College

Xixin He
School of Pharmaceutical Science, Guangzhou University of Chinese Medicine

Chuan Bai
Institute of Human Virology, Department of Biochemistry and molecular biology, Zhongshan School of Medicine, Sun Yat-sen University

Zhaofeng Huang (hzhaof@mail.sysu.edu.cn)
Institute of Human Virology https://orcid.org/0000-0003-4367-6943

Research article

Keywords: RORγt, Th17, lupus nephritis, thymocyte development

Posted Date: February 7th, 2020

DOI: https://doi.org/10.21203/rs.2.22921/v1

Version of Record: A version of this preprint was published at Immunologic Research on July 4th, 2021.
See the published version at https://doi.org/10.1007/s12026-021-09204-5.

Abstract

Objective To investigate the effect of a RORγt inhibitor, 5,6,7,8-tetrahydrobenzo [4,5]thieno [2,3-d]pyrimidine derivative (TTP), on the therapy of lupus nephritis and the safe risk on thymocytes development. Methods BALB/C female mice were 2 months for pristane-induced mice model of lupus nephritis with the treatment of vehicle, prednisone acetate, and TTP. And 4-week-old BALB/C female mice were used for studying the development of thymus with the treatment of vehicle and TTP. Results TTP repressed the development of Th17 cells and ameliorated the autoimmune disease manifestation in a pristine induced lupus nephritis mouse model. The treatment of TTP in mice didn't interfere with thymocyte development, including total thymocyte numbers and proportion of CD4+CD8+ double-positive populations in thymus, and had no substantial effects on thymoma incidence. Surface plasmon resonance identified the TTP had stronger affinity with full length RORγt protein compared the truncated RORγt LBD region, indicated TTP binding to RORγt beyond LBD region. Molecular docking computation showed the best binding pocket of TTP to RORγt located in the hinge region of RORγt. Conclusion As a RORγt inhibitor, TTP had potential to develop drug for treating Th17-cell-mediated autoimmune diseases with low safety risk for thymocyte development.

1 Introduction

Systemic lupus erythematosus (SLE) is a chronic autoimmune driven by multiple productions of self-antibody, such as anti-dsDNA antibodies which are a canonical parameter and diagnostic criteria in clinical[1] and antinuclear antibodies (ANAs)[2]. All of these self-antibodies are important for pathology of lupus nephritis (LN) which is the key cause of death for SLE[2]. Th17 cells, a novel effect T cell subset producing IL-17A[3], have an important role in the pathology of autoimmune[3–6]. IL-17A, as a pro-inflammation cytokine[7], has a central role in pathogenesis of LN[8, 9]. IL-17A is abnormally elevated in multiple autoimmune models in murine, such as SLE, rheumatoid arthritis (RA)[10] and another lupus model[11]. Recently, treating psoriasis and rheumatoid arthritis via inhibiting the function of Th17 cells are reported and several drugs have been approved in the clinical application[12, 13].

RORγt, a member of retinoic-acid-receptor-related orphan nuclear hormone receptor (ROR) family[14], extensively express in immune system[15, 16]. It is essential for the positive selection of thymocytes and CD4 + CD8+ (double positive, DP) thymocytes' survival[17]. Previously study revealed that Rorγt-deficient mice could defect on T cell development, increase the apoptotic thymocytes and alter the proportion of CD4/CD8 subpopulations in 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 a AF2 domain to recruit SRC family of co-activators stimulating gene expression[21], and a hinge domain (HD) link DNA-binding domain with ligand-binding domain. Previously studies showed that RORγt inhibitors prevented the development of Th17 cell-dependent autoimmunity, as well as interfered T cell development in thymus[22, 23]. Recent studies indicated that some regions in the hinge domain required for suppression of Th17 cells differentiation but not for the development of thymus and lymph node[24]. It suggested that HD of RORγt was a target for specifically inhibiting Th17 cells function.

RORγt is a master transcription factor for Th17 cells differentiation[25] and affects the function of Th17 cells by inducing the expression of IL-17A gene and IL-17F gene[25]. RORγt-deficient T cell displayed a significant reduction in the ability to differentiate Th17 cell, 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 can inhibit the activity of RORγt, block Th17 differentiation, and ameliorate autoimmune disease manifests[27, 29]. In previous studies, we found that tetraazacyclic compounds have a potentiality to inhibit RORγt transcription ability[30]. 5,6,7,8-tetrahydrobenzo [4, 5]thieno [2,3-d]pyrimidine derivative(TTP) belongs to the tetraazacyclic compounds family, and display excellent performance on RORγt activity inhibition, so it has been chosen as the target compounds for this study. The structure of TTP was described in article 41 [31] (Fig. 1a). In this study, we further reveal that TTP inhibition on the subset T cell differentiation. Furthermore, we also demonstrate that TTP attenuates the manifest of lupus nephritis. We investigate the effect of TTP on thymic development and observe the effect on the incidence of T cell lymphoma. Surface plasmon resonance was employed to the affinity of TTP to RORγt protein, as well as the Molecular docking computation to reveal the binding pocket of TTP to RORγt. With these results, we evaluate the potential of TTP as the lead compound for drug discovery in Th17 mediated autoimmune disease therapy.

2 Materials And Methods

2.1 Mice.

Two months old C57B6/J female mice, 8 -week-old BALB/C female mice and 4-week-old BALB/C female mice were purchased from 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 8 weeks for T cell differentiation in vitro, BALB/C female mice were 2 months for pristane-induced mice model of lupus nephritis and 4-week-old BALB/C female mice were used for studying the development of thymus.

2.2 T cell differentiation in vitro.

Naïve CD4 + T cells were purified from C57B6/J mice by a MACS magnetic column with a CD4 + T cell negative enrichment kit according to the manufacturer's protocol (eBioscience, USA). Suspension of 1 × 10^6 cells/ml RPIM-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 supplemented with cytokines: 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; 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. After three days of culture, cells were incubated for 6 h with 50 ng/ml PMA (Sigma-Aldrich), 1 μg/ml ionomycin (Sigma-Aldrich) and 1 μg/ml brefeldin A (BD Biosciences) in a tissue culture incubator at 37 °C, and then by flow cytometry analysis.

2.3 Apoptosis assay.

Thymocytes were freshly isolated from 8 -week-old C56B6/J mice cultured in RPMI 1640 medium supplemented with 10%FBS, 100 U/ml penicillin–streptomycin, and 2 mM L-glutamine at 1 × 10^6 cells/ml. Cells were cultured with different concentration of TTP, including 0.5 μM, 1 μM, 2 μM, 4 μM, 8 μM. Then those cells were incubated at 37 °C with 5% CO2. Annexin V-FITC (ebioscience, Cat. No. 11-8005-74) and PI staining (ebioscience, Cat. No. 00-6990-42) analysis for dead cells at the following time points: 2 h, 4 h, 6 h, 9 h for thymocytes.

2.4 Mice model with lupus nephritis induced by pristane.

8 -week-old Balb/c female mice were induced to lupus nephritis by intraperitoneal injection of 500 μl pristane (Sigma-Aldrich). Control mice were received an equal volume of saline at the same way injection (n = 6). At the 6 months old mice were treated with drugs, including prednisone acetate and TTP. The grouping is following: (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); (3) the TTP-treated group treated with TTP (15 mg/kg, n = 16). All of the drugs were dissolved with 25% ethanol and 75% cyclodextrin and each mouse was intragastric administration with 100 μl drugs twice a week for two months.

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

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

2.6 Renal pathology and immune complex deposition

2.6.1 Pathological analysis of the kidney.

Kidneys were soaked in 4% Polyoxymethylene for one day, embedded in paraffin, cut at 5 μm thickness. Sections were stained with Periodic acid-Schiff stain (PAS)

2.6.2 Detection of renal immune complex deposition.

Kidney sections were stained with Alexa Fluor 488 goat anti-mouse IgM (μ chain). The antibody was diluted at 1:200. Evaluation of the depositions of immune complexes of IgM in glomeruli were 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).

2.7 Thymic development of mice.

4-week-old BALB/C mice were treated with 25% ethanol and 75% cyclodextrin (vehicle group, n = 10) and TTP (TTP group, n = 10), and health group (n = 8) with not any treatment. To 8 weeks, collecting thymus and spleen of mice to detect the proportion of cells, the development of thymus, and the apoptosis of thymocytes. The apoptosis of thymocytes were detected by In Situ Cell Death Detection Kit, TMR red (Roche, Cat. No. 12156792910). The positive cells in randomly five views were detected by a fluorescence microscope (Lump, Japan). The ratio of apoptotic cells was calculated as positive cells/total cells. To 12 weeks, collecting thymus and spleen of mice to detect the proportion of lymphocytes in spleen and the T cell lymphoma development in the thymus. The T cell lymphoma development in the thymus was detected by H&E staining.

2.8 Flow cytometry

The flow cytometry analysis differentiation of T cell by following fluorescent antibodies: 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), Alexa flour 680 anti-mouse Foxp3 (BD, Cat. No. 560401). Data were analyzed by FlowJo software (FlowJo VX, BD). The flow cytometry analysis proportion of cells in spleen or thymus by following fluorescent antibodies: 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), FITC anti-mouse B220 (eBioscience, Cat. No. 11-0452-82).

2.9 RNA isolation and Quantitative real-time PCR

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

The primers were used in qRT-PCR as following: GAPDH, forward sequence: TGGTGAAGGTCGGTGTGAAC, reverse sequence: CCATGTAGTTGAGGTCAATGAAGG; RORγt, forward sequence: AAACTTGACAGCATCTCGGGA, reverse sequence: TGTAATGTGGCCTACTCCTGCA; IL-17A, forward sequence: TTTAACTCCCTTGGCGCAAAA; reverse sequence: CTTTCCCTCCGCATTGACAC; IL-17F, forward sequence: GAGGATAACACTGTGAGAGTTGAC, reverse sequence: GAGTTCATGGTGCTGTCTTCC; T-bet: forward sequence: AACACACACGTCTTTACTTTCCA, reverse sequence: CGTATCAACAGATGCGTACATGG; IFN-γ: forward sequence: GCCACGGCACAGTCATTGA, reverse sequence: TGCTGATGGCCTGATTGTCTT; Foxp3: forward sequence: GGCCCTTCTCCAGGACAGA, reverse sequence: GCTGATCATGGCTGGGTTGT; helios: forward sequence: GAGCCGTGAGGATGAGATCAG, reverse sequence: CTCCCTCGCCTTGAAGGTC.

2.10 SPR

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 pH7.9, 1.5 mM MgCl2, 420 mM NaCl, 0.2 mM EDTA, 0.5 mM Dithiothreitol (DTT), 25% glycerol) and ultrasonication. The proteins were purified on HisPur Cobalt Resin (Thermo scientific, Cat No. 89965). Full-length RORγt protein and RORγt ligand binding domain 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(Biocore T100, Sweden GE).

2.11 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.

2.12 Statistical analysis

Prism software (GraphPad 7.0) was used for all statistical analyses. A p-value of less than 0.05 was considered statistically significant. Data are expressed as mean ± SEM.

3 Results

3.1 TTP specifically inhibits Th17 cells differentiation in vitro

We have discovered that TTP is a RORγt inhibitor in the previously reported[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. 1b, c). The IL-17A and IL-17F were significantly inhibited, rather than RORγt expression (Fig. 1. c). TTP also inhibited Th1 differentiation with the reduced IFN-γ secretion and the expression T-bet (Fig. 1d, e). We observed no effect on the Treg differentiation with the same expression level of Foxp3 and helios (Fig. 1f, g).

3.2 Alleviation the pathogen manifest of lupus nephritis in the mice

1) Reduced serum dsDNA level in lupus nephritis mice

In order to evaluate the therapeutic potential of TTP on Th17-related autoimmune diseases, we investigated the effect of TTP on lupus nephritis mice induced by pristane. In this study, we induced mouse model of lupus nephritis by injected pristane into 8-week-old mice. In the sixth month of mice (4 months after injecting pristane), those were treated with TTP twice per week. Serum anti-dsDNA antibody levels were examined at the following time points:2 months of age (before pristane injection), 6 months (starting point of TPP treatment), 7 months (one month after TTP treatment) and 8 months (two months after TTP treatment). The result display that serum anti-dsDNA antibody levels had dramatically increased in the model group at 7 and 8 months compared with the control group (Fig. 2a). Nevertheless, serum anti-dsDNA antibody levels in the TTP-treated group were dramatically decreased compared to the model group, similar to the positive drug treatment group with prednisone acetate (Fig. 2a, 2b).

2) TTP can alleviate renal damage in mice with lupus nephritis

We next assessed pathological changes of the kidney via PAS stained, and found that the volume of the glomerulus in mice with lupus nephritis was enlarger than the control group, lymphocytes infiltration enhanced and capillary hyperplasia (Fig. 2c, middle). However, it was remission after TTP-treated (Fig. 2c, right) and clinical histopathological scores were significantly decreased (Fig. 2e). And then, immunofluorescence analysis the deposition of anti-IgM antibody in the kidney. We observed that the increasement of anti-IgM antibody in lupus nephritis mice compared with the control group was reduced after TTP-treated (Fig. 2d, 2e). It indicated that TTP could alleviate renal damage with the reduction of immune complex accumulation in lupus nephritis mice.

3) TTP slightly inhibits Th17 cells in the spleen

To further determine the effect of TTP on Th17 function in lupus nephritis mice, we detected the Th17 cells (Fig. 3a) and Th1 cells (Fig. 3b) in the spleen. These cells have slightly reduced under the treatment of TTP. Th17-related signature genes, including IL17A (Fig. 3c), IL17F (Fig. 3d), were all slightly reduced in the presence of TTP.

3.3 No interference with thymic development of mice

Previously studies indicated that RORγt regulated thymocyte survival and development of thymus, therefore, we further studied whether TTP had the same effects in the development of thymus. We treat 4-week-old mice with TTP for 4 weeks period. The results showed that total cell numbers in thymus have no significant difference with treatment of TTP (Fig. 4a), as well as in the population of different thymocytes, CD4 + CD8 + double-positive, CD4 + single positive, CD8 single positive, and CD4-CD8-double negative thymocytes (DN) were no difference among the treated group, health group and vehicle group (Fig. 4b, 4c).

We also investigated whether TTP has an effect on other peripheral immune organs. The frequency of lymphocytes in the spleen, as well as the proportion of T cells and B cells, had no significant difference between TTP-treated groups and health groups or vehicle-treated group (Fig. 4d, e). In total T cells, the proportion of CD4 + and CD8 + T cells also unaffected under treatment of TTP for 4weeks (Fig. 4f, 4 g).

3.4 No effect on thymocytes apoptosis and thymic lymphoma

RORγt is a crucial transcription factor for preventing thymocytes apoptosis, and defect thymocytes shown accelerated spontaneous apoptosis in the previous study. In this study, we explored whether TTP has an influence on thymocytes' apoptosis. The results showed that TTP only slightly increased thymocytes' apoptosis at high concentrations (Fig. 5a and 5b). We also investigated the effect of TTP in the thymus with TUNEL assay to detect the thymocyte apoptotic cell in vivo. TTP increased very small proportion of apoptotic thymocytes compared with vehicle or health groups (less than 1%) (Fig. 5c, d), The apoptotic frequency of thymocytes were 7.4%, 7.8% and 8.3% in health, vehicle and TTP-treated groups, respectively (Fig. 5d).

Nevertheless, the lymphoma did not produce in the thymus of the mouse with TTP-treated for 8 weeks (Fig. 5e). Thymus had a normal cortical/medullary (Fig. 5e-left and middle) and did not the presence of larger thymocytes (Fig. 5e-right) following 8-week treatment with TTP. And then, numerical and flow cytometric analysis revealed no difference in numbers of total thymocytes and frequency of thymocytes population (data not shown). These results indicated that TTP has no obvious side effects on thymocyte development and thymoma pathogenesis.

3.5 Interaction with hinge domain of RORγt

We next studied the binding affinity of TTP with RORγt protein via SPR, with which TTP demonstrated stronger binding affinity with full-length RORγt protein (Fig. 6a) compared with RORγt ligand binding domain (LBD) protein (Fig. 6b), with the KD 44.1 μM vs 99.4 μM respectively. With molecular docking computation, the best binding pocket for TTP with RORγt protein 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 may interact with the hinge domain to regulate RORγt transcription activity.

4 Discussion

Systemic lupus erythematosus (SLE) is a chronic disease and involves virtually every organs and tissues, including kidney, known as lupus nephritis (LN), which is a major cause of morbidity and mortality in SLE patients[32, 33]. The lupus nephritis patients have many representative clinical symptoms, such as increased anti-dsDNA antibody level in serum, infiltration of lymphocytes and immune-complex deposited in the kidney. The IgM and IgG complement-fixing antibodies to dsDNA deposited in the kidney relating to the risk of glomerulonephritis[34].So far, the therapy of SLE is very challenging due to the etiology of the disease remains elusive. It is generally used in combination with a variety of therapies in clinical. Immunosuppressive agents such as azathioprine (AZA), cyclophosphamide (CYC) and chlorambucil were administrated either individually or combining with each other or corticosteroids for treating lupus nephritis[35–38]. However, these agents worked on many immune cells with severe side effects, which

limited the application in SLE. Increasing pieces of evidence indicate the critical role of IL-17A and Th17 cells in SLE and other human autoimmune diseases [3–6], and they are an essential role in the pathogenesis of lupus nephritis[8, 9, 39]. In this report, we observed that the levels of anti-dsDNA antibody in serum were significantly decreased after treating TTP for 2 months (Fig. 2a, b), and the deposition of IgM immune-complex in kidney markedly decreased with TTP treatment (Fig. 2d, f). These demonstrated that TTP could ameliorate the clinical manifestation of lupus nephritis in mice, indicating its application in human SLE therapy. RORγt, a central transcription factor in Th17 cells differentiation, had a treating potential for Th17-derived autoimmune inflammation as a therapeutic target[40, 41]. However, RORγt also is mainly expressed in DP thymocytes subpopulations[42, 43], and Rorc-deficient (Rorc-/-) in mice would increase thymocyte apoptosis, alter CD4/CD8 subpopulation in the thymus, leading to the emergence of T lymphoma in the thymus[17, 18, 41, 44, 45]. Generous studies showed that small molecule agents as RORγt inhibitors had an inhibition for the functions of Th17 cells[22, 27, 28, 46], such as SR1001, digoxin, and ML209, but had others side effects in thymus, including DP thymocytes apoptosis, anti-apoptotic genes downregulated and the induction thymic lymphoma [23, 47]. In this study, we found that TTP disturbed the functions of Th17 cells, however, it did not affect the numbers and frequency of CD4 + CD8 + DP thymocytes population, and the total numbers of thymocytes had no significant difference with control group (Fig. 4a, b). Similarly, the subpopulations of T lymphocytes in spleen had no difference in treating TTP after 4-weeks and 8-weeks (data not shown). We also observed no increase in large thymocytes in mice after 8 weeks treated with TTP, indicating no obvious change of thymoma incidence (Fig. 5e). However, it should be to treat for a longer period to confirm this observance about the incidence of lymphoma. Although most evidences about thymocyte development displayed normal, high concentration TTP could increase the speed of apoptosis with the in vitro thymocytes culture (Fig. 5a, b), and proportion of the DNA damaged cell in thymus, which detected by TUNAL assay, had slightly increased after TTP treatment (Fig. 5c, d), indicating low safety risk of TTP on thymocyte development.

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 a AF2 domain to recruit SRC family of co-activators stimulating gene expression[21]; and a hinge domain (HD) link DNA-binding domain with ligand-binding domain. Previously study indicated that mutation of AF2 of RORγt interfered the ability of active target genes and recruitment of coactivators[48]. However, disruption of the functions of LBD and DBD of RORγt inhibits the IL-17A-mediated autoimmune inflammation, as well as the thymocytes' survival[21, 48]. Previously study showed that two amino acid mutations in HD of RORγt were suppressing the functions of Th17 cells but not disrupted thymocyte survival[23]. HD region probably plays a crucial role in discrimination of RORγt function between Th17 differentiation and thymocyte development. In this hypothesis, compound interacted with the HD region of RORγt may specifically affect Th17 differentiation, rather than thymocyte development. In this study, we demonstrated that TTP had a strong affinity with full-length RORγt, rather than the RORγt LBD region, indicating it may bound to HD of RORγt (Fig. 6a, b). Molecular docking computational analysis also demonstrated the best pocked for TTP binding to RORγt located in the HD region. Therefore, the HD of RORγt may be the target region of

TTP. It remains to describe the mechanism of TTP regulating RORγt function by interacting with the HD region.

Other T cell subsets also contribute to autoimmune disease pathogenesis. Treg cells, as the major suppressive immune cells, were very important to maintain immunologic self-tolerance as well as prevent autoimmune diseases[49]. Notably, the balance between Treg cells and Th17 cells is crucially involved in autoimmune diseases. However, we didn't observe TTP affect Treg cells in this study(Fig. 1f, g)indicated Treg were not the targets of TTP. Some autoimmune disease etiologies were also attributed to Th1 cells, including multiple sclerosis, rheumatoid arthritis, and SLE[50]. In this study, TTP also suppressed Th1 differentiation in vitro and decreased the numbers of CD4 + IFN-γ + cells in the LN mouse model (Fig. 1d, 1e and Fig. 3b). These results indicated that Th1 cells could be the alternative pathway of TTP, coordinated with Th17 cells to alleviate LN pathogenesis. More efforts need to conduct for revealing the mechanism of TTP regulating Th1 function. Moreover, the cooperation of manipulating Th1 and Th17 function via TTP remains to describe in the future study.

5 Conclusions

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

Abbreviations

ELISA:Enzyme-linked immunosorbent assay; qRT-PCR:Quantitative real-time polymerase chain reaction; SPR:Surface plasmon resonance; PAS:Periodic acid-Schiff; IgM:Immunoglobulin M; SLE:Systemic lupus erythematosus; LN:Lupus nephritis; PA:prednisone acetate; IFN-γ:Interferon gamma ; RORγt:retinoid-related orphan nuclear receptor gamma t ; IL-17A:Interleukin 17A ; IL-17F:Interleukin 17F ; GAPDH:Glyceraldehyde-3-phosphate dehydrogenase; Th1:T helper cell 1 ; Th17:T helper cell 17; Treg:Regulatory cell;

Declarations

Acknowledgements

Not applicable

Authors' contributions

H.Z and B.C design of the study. W.F, Z.X, C.H, T.X, H.X, Y.B, and B.C performed experiments, collected and analyzed data. L.Z provided technical support on the mouse model. W.F, B.C, and H.Z drafted the manuscript. All authors revised and approved the manuscript.

Funding

This research was supported by the Science and Technology Department of Guangdong province (2017A050501010, 2017A020211003, and 2016A050503023), Guangzhou Science Technology and Innovation Commission 201807010042 and 201704030087 National Natural Science Foundation of China (21572281, 21877132)

Availability of data and materials

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

Ethics approval and consent to participate

All animal protocols were approved by the Hospital Research Ethics Committee

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

References

1. Tan EM, Cohen AS, Fries JF, Masi AT, McShane DJ, Rothfield NF, Schaller JG, Talal N, Winchester RJ: The 1982 revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum 1982, 25:1271-1277.
2. Rekvig OP: Anti-dsDNA antibodies as a classification criterion and a diagnostic marker for systemic lupus erythematosus: critical remarks. Clin Exp Immunol 2015, 179:5-10.
3. Harrington LE, Hatton RD, Mangan PR, Turner H, Murphy TL, Murphy KM, Weaver CT: Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. Nat Immunol 2005, 6:1123-1132.
4. Matusevicius D, KivisaKk P, He B, Kostulas N, Ozenci V, Fredrikson S, Link H, . %J Mult Scler: Interleukin-17 mRNA expression in blood and CSF mononuclear cells is augmented in multiple sclerosis. Mult Scler 1999, 5:101-104.
5. Fujino S, Andoh A, Bamba S, Ogawa A, Hata K, Araki Y, Bamba T, Fujiyama Y, .%J Gut: Increased expression of interleukin 17 in inflammatory bowel disease. Gut 2003, 52:65-70.
6. Lubberts E, Koenders MI, Berg WBVDJAR, Therapy: The role of T-cell interleukin-17 in conducting destructive arthritis: lessons from animal models. Arthritis Res Ther 2005, 7:29-37.
7. Kolls JK, Linden A: Interleukin-17 family members and inflammation. Immunity 2004, 21:467-476.

8. Zhang Z, Kyttaris VC, Tsokos GC: The role of IL-23/IL-17 axis in lupus nephritis. J Immunol 2009, 183:3160-3169.
9. Apostolidis SA, Crispin JC, Tsokos GC: IL-17-producing T cells in lupus nephritis. LUPUS 2011, 20:120-124.
10. Biswas PS, Kang K, Gupta S, Bhagat G, Pernis AB: A murine autoimmune model of rheumatoid arthritis and systemic lupus erythematosus associated with deregulated production of IL-17 and IL-21. Methods Mol Biol 2012, 900:233-251.
11. Wen Z, Xu L, Xu W, Yin Z, Gao X, Xiong S: Interleukin-17 expression positively correlates with disease severity of lupus nephritis by increasing anti-double-stranded DNA antibody production in a lupus model induced by activated lymphocyte derived DNA. PLoS One 2013, 8:e58161.
12. Genovese MC, Van den Bosch F, Roberson SA, Bojin S, Biagini IM, Ryan P, Sloan-Lancaster J: LY2439821, a humanized anti-interleukin-17 monoclonal antibody, in the treatment of patients with rheumatoid arthritis: A phase I randomized, double-blind, placebo-controlled, proof-of-concept study. Arthritis Rheum 2010, 62:929-939.
13. Leonardi C, Matheson R, Zachariae C, Cameron G, Li L, Edson-Heredia E, Braun D, Banerjee S: Anti-interleukin-17 monoclonal antibody ixekizumab in chronic plaque psoriasis. N Engl J Med 2012, 366:1190-1199.
14. Jetten AM: Recent advances in the mechanisms of action and physiological functions of the retinoid-related orphan receptors (RORs). Curr Drug Targets Inflamm Allergy 2004, 3:395-412.
15. Eberl G, Littman DR: The role of the nuclear hormone receptor RORgammat in the development of lymph nodes and Peyer's patches. Immunol Rev 2003, 195:81-90.
16. Egawa T, Eberl G, Taniuchi I, Benlagha K, Geissmann F, Hennighausen L, Bendelac A, Littman DR: Genetic evidence supporting selection of the Valpha14i NKT cell lineage from double-positive thymocyte precursors. Immunity 2005, 22:705-716.
17. Sun Z, Unutmaz D, Zou YR, Sunshine MJ, Pierani A, Brenner-Morton S, Mebius RE, Littman DR: Requirement for RORgamma in thymocyte survival and lymphoid organ development. Science 2000, 288:2369-2373.
18. E U, S K, M S, M B, B K, AM J: High incidence of T-cell lymphomas in mice deficient in the retinoid-related orphan receptor RORgamma. Cancer Res 2002, 62:901.
19. Langrish, Medicine CLJJoE: IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. J Exp Med 2005, 201:233-240.
20. Sherlock JP, Joyce-Shaikh B, Turner SP, Chao C-C, Sathe M, Grein J, Gorman DM, Bowman EP, McClanahan TK, Yearley JHJNM: IL-23 induces spondyloarthropathy by acting on ROR-γt+ CD3+CD4-CD8- entheseal resident T cells. Nat Med 2012, 18:1069-1076.
21. Xie H, Huang Z, Wang R, Sun ZJCRiI: Regulation of Thymocyte Survival by Transcriptional Coactivators. Crit Rev Immunol 2006, 26:475-486.
22. Huh JR, Littman DR: Small molecule inhibitors of RORγt: Targeting Th17 cells and other applications. Eur J Immunol 2012, 42:7.

23. Guntermann C, Piaia A, Hamel ML, Theil D, Schümann JJJI: Retinoic-acid-orphan-receptor-C inhibition suppresses Th17 cells and induces thymic aberrations. JCI Insight 2017, 2:e91127.
24. He Z, Ma J, Wang R, Zhang J, Huang Z, Wang F, Sen S, Rothenberg EV, Sun Z: A two-amino-acid substitution in the transcription factor RORγt disrupts its function in TH17 differentiation but not in thymocyte development. Nat Immunol 2017, 18:11.
25. Ivanov, II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, Lafaille JJ, Cua DJ, Littman DR: The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. Cell 2006, 126:1121-1133.
26. Huh JR, Leung MW, Huang P, Ryan DA, Krout MR, Malapaka RR, Chow J, Manel N, Ciofani M, Kim SV *et al*: Digoxin and its derivatives suppress TH17 cell differentiation by antagonizing RORgammat activity. Nature 2011, 472:486-490.
27. Solt LA, Kumar N, Nuhant P, Wang Y, Lauer JL, Liu J, Istrate MA, Kamenecka TM, Roush WR, Vidovic D *et al*: Suppression of TH17 differentiation and autoimmunity by a synthetic ROR ligand. Nature 2011, 472:491-494.
28. Xiao S, Yosef N, Yang J, Wang Y, Zhou L, Zhu C, Wu C, Baloglu E, Schmidt D, Ramesh R *et al*: Small-molecule RORgammat antagonists inhibit T helper 17 cell transcriptional network by divergent mechanisms. Immunity 2014, 40:477-489.
29. SM B, JR Z, JJ C: RORγ antagonists and inverse agonists: a patent review. Expert Opin Ther Pat 2016, 27:1.
30. Ding Q, Zhao M, Yu B, Bai C, Huang Z: Identification of Tetraazacyclic Compounds as Novel Potent Inhibitors Antagonizing RORgammat Activity and Suppressing Th17 Cell Differentiation. PLoS One 2015, 10:e0137711.
31. Lao C, Zhou X, Chen H, Wei F, Huang Z, Bai C: 5,6,7,8-Tetrahydrobenzo[4,5]thieno[2,3-d]pyrimidine derivatives as inhibitors of full-length RORgammat. Bioorg Chem 2019, 90:103077.
32. Maroz N, Segal MSJAJotMS: Lupus Nephritis and End-stage Kidney Disease. Am J Med Sci 2013, 346:319-323.
33. Bertsias GK, Boumpas DT: Connective tissue diseases: Lupus nephritis-winning a few battles but not the war. Nat Rev Rheumatol 2011, 7:441-442.
34. Hahn BH: Antibodies to DNA. N Engl J Med 1998, 338:1359.
35. Grupe WE, Heymann W: Cytotoxic drugs in steroid-resistant renal disease. Alkylating and antimetabolic agents in the treatment of nephrotic syndrome, lupus nephritis, chronic glomerulonephritis, and purpura nephritis in children. Am J Dis Child 1966, 112:448-458.
36. White RH, Cameron JS, Trounce JRJBMJ: Immunosuppressive therapy in steroid-resistant proliferative glomerulonephritis accompanied by the nephrotic syndrome. Br Med J 1966, 2:853-860.
37. Maher JFJAoIM: Treatment of Lupus Nephritis With Azathioprine. Archives of internal medicine 1970, 125:293.
38. Adams, Jama DAJ: Azathioprine Treatment of Immunological Renal Disease. JAMA 1967, 199:459.

39. Aggarwal S, Ghilardi N, Xie MH, de Sauvage FJ, Gurney AL: Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. J Biol Chem 2003, 278:1910-1914.
40. Huang Z, Xie H, Wang R, Sun ZJEOoTT: Retinoid-related orphan receptor γt is a potential therapeutic target for controlling inflammatory autoimmunity. Expert Opin Ther Targets 2007, 11:737-743.
41. Jetten AM, Ueda E: Retinoid-related orphan receptors (RORs): roles in cell survival, differentiation and disease. Cell Death Differ 2002, 9:1167-1171.
42. He YW, Beers C, Deftos ML, Ojala EW, Forbush KA, Bevan MJJJoI: Down-regulation of the orphan nuclear receptor RORγt is essential for T lymphocyte maturation. J Immunol 2000, 164:5668-5674.
43. Ortiz MA, Piedrafita FJ, Pfahl M, Maki R: TOR: a new orphan receptor expressed in the thymus that can modulate retinoid and thyroid hormone signals. Mol Endocrinol 1995, 9:1679-1691.
44. Kurebayashi S, Ueda E, Sakaue M, Patel DD, Medvedev A, Zhang F, Jetten AMJPotNAoSotUSoA: Retinoid-related orphan receptor gamma (RORgamma) is essential for lymphoid organogenesis and controls apoptosis during thymopoiesis. Proc Natl Acad Sci U S A 2000, 97:10132-10137.
45. Maria, Liljevald, Maria, Rehnberg, Magnus, Söderberg, Marie, Ramnegård, Jenny, Börjesson: Retinoid-related orphan receptor γ (RORγ) adult induced knockout mice develop lymphoblastic lymphoma. Autoimmun Rev 2016, 15:9.
46. Huh JR, Littman DR: Small molecule inhibitors of RORgammat: targeting Th17 cells and other applications. Eur J Immunol 2012, 42:2232-2237.
47. Imura C, Ueyama A, Sasaki Y, Shimizu M, Furue Y, Tai N, Tsujii K, Katayama K, Okuno T, Shichijo M *et al*: A novel RORgammat inhibitor is a potential therapeutic agent for the topical treatment of psoriasis with low risk of thymic aberrations. J Dermatol Sci 2019, 93:176-185.
48. Xie H, Sadim MS, Sun Z: RORgammat recruits steroid receptor coactivators to ensure thymocyte survival. J Immunol 2005, 175:3800-3809.
49. Sakaguchi S: Naturally Arising CD4+ Regulatory T Cells for Immunologic Self-Tolerance and Negative Control of Immune Responses. Annu Rev Immunol 2004, 22:531-562.
50. Zhu J, Paul WEJB: CD4 T cells: fates, functions, and faults. Blood 2008, 112:1557-1569.

Figures

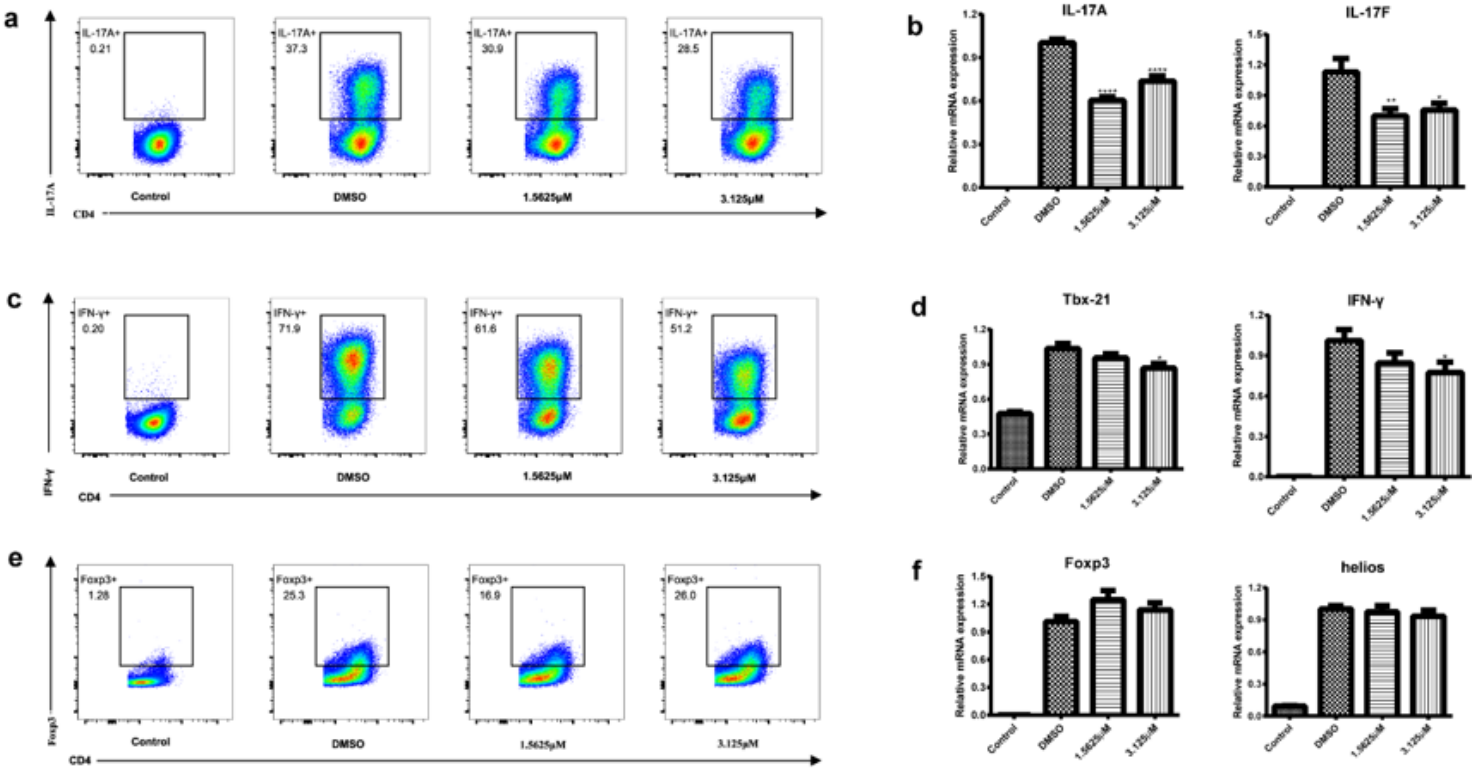

Figure 1

TTP inhibits Th17 differentiation in vitro. The structure of TTP was shown (a). Naive CD4+ T cells were sorted from wild-type C57B6/J mice and were differentiated into T helper cells with differentiation concentrations of TTP. Flow cytometric analysis of IL-17A expression (b), IFN-γ (d), and Foxp3 (f) in naïve CD4+ T cells differentiated under Th17 cells, Th1 cells and Treg cells priming conditions, respectively. RT-qPCR analysis of Il17A, Il17F (c) and T-bet, IFN-γ (e), Foxp3, helios (g) mRNA in WT Th17 cells, Th1 cells and Treg cells, respectively. The in vitro differentiation and quantitative real-time PCR were repeated 3 times with consistent results and the results are quantified and normalized to GAPDH expression (n=3). The results are shown as mean ± SEM; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

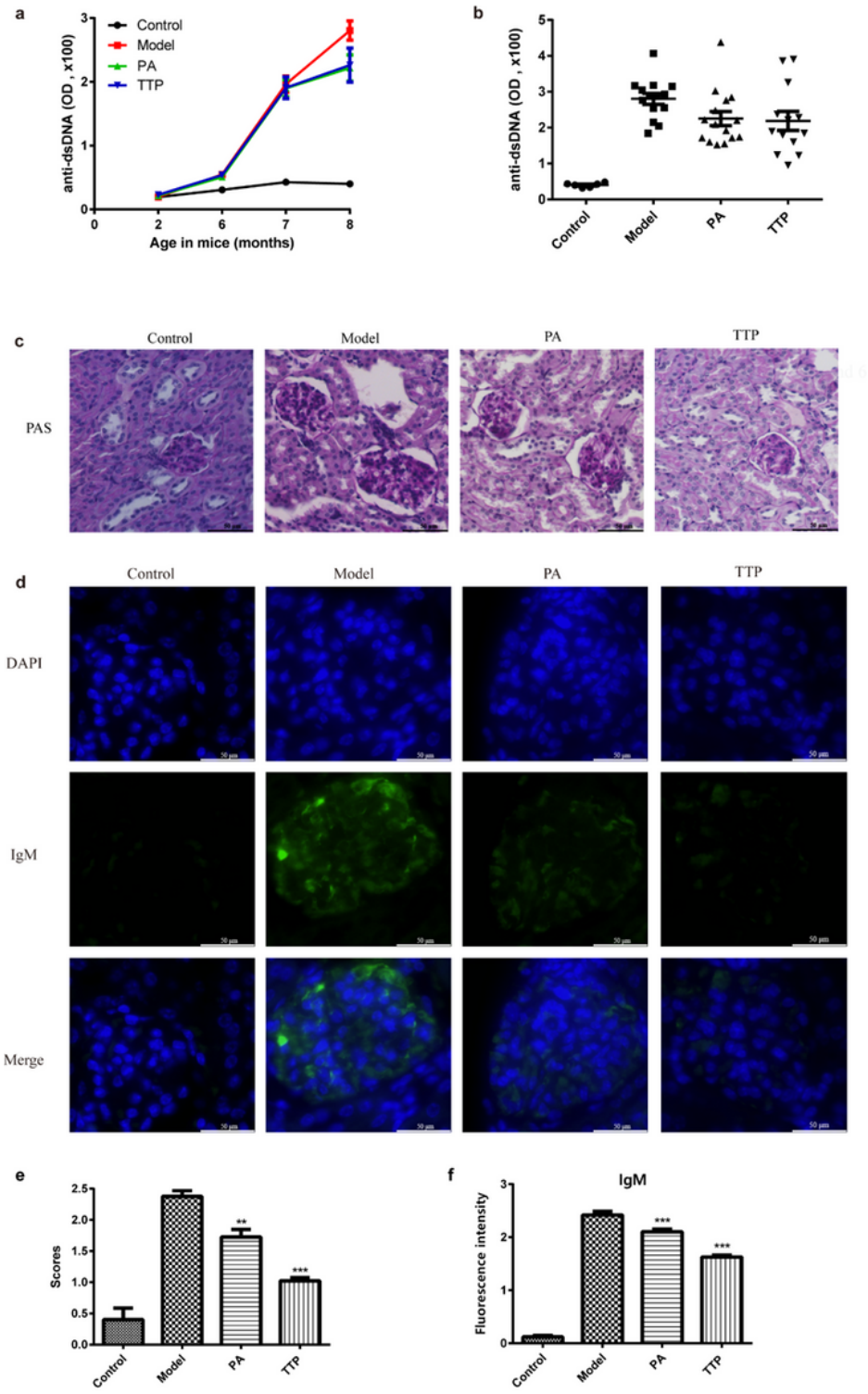

Figure 2

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 of after pristane injection), 7 months (one month after TTP treatment-the endpoint of experiment), 8 months (two months after TTP treatment-the endpoint of 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 (c and e) and

immunofluorescence (d and f) analyzed renal damage. PAS-stained detected the volume of glomeruli and infiltration of lymphocytes (c) and clinical histopathological scores were shown according to PAS stained (e). The immunofluorescence analysis anti-IgM antibody in the kidney (d) and fluorescence intensity of IgM deposition are shown, 15-20 glomeruli were examined and the average scores were obtained. Control group (n=6), model group (n=14), positive drugs-prednisone acetate (PA) (n=15), TTP-treated group (n=12). Scale bar=50μm. Data are presented as mean ± SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

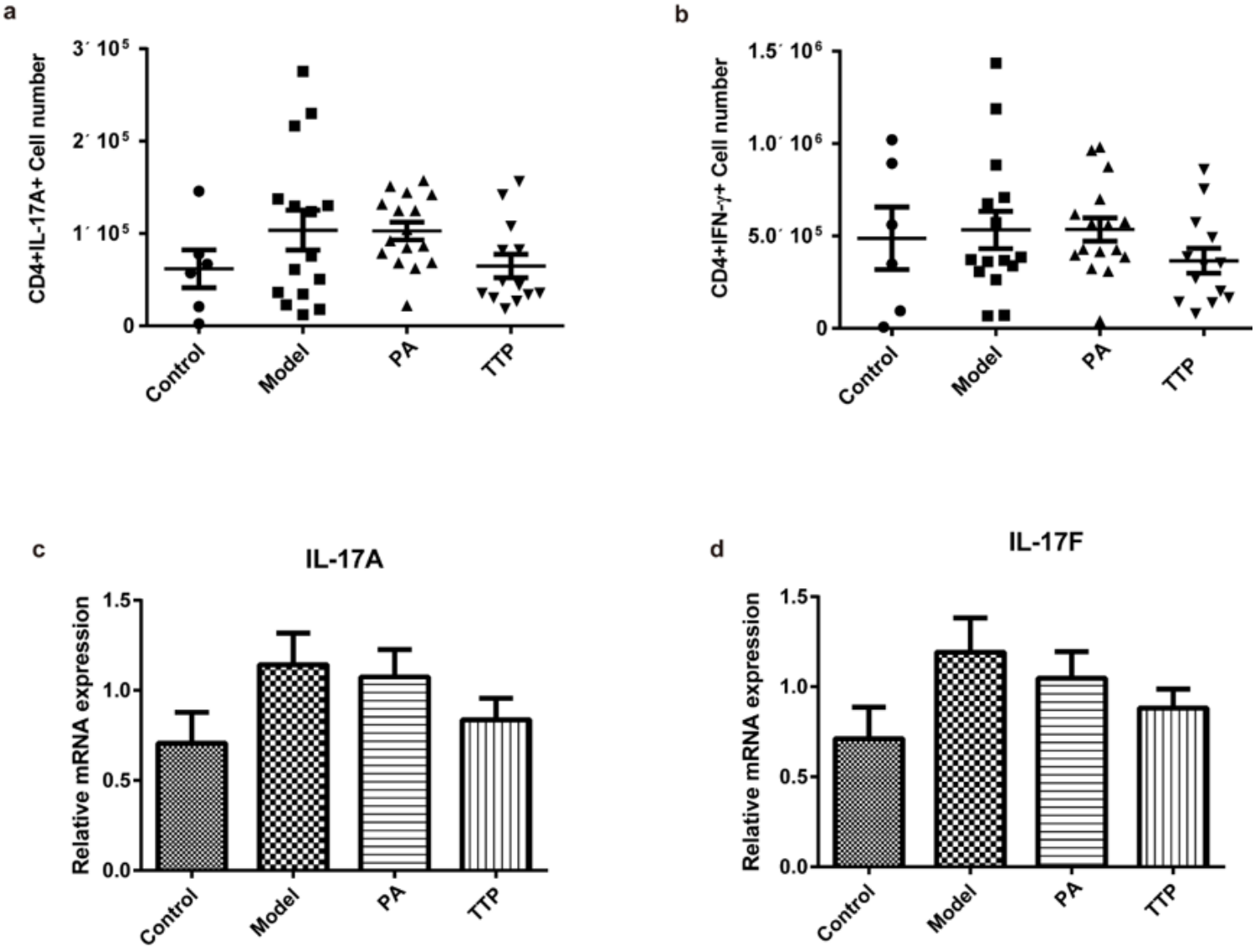

Figure 3

TTP could inhibit functions of Th17 cells in mice with lupus nephritis. Spleen lymphocytes were collected from control, the model control group, prednisone acetate-treated group (PA) (15mg/kg) and TTP-treated group (15mg/kg) at 8 months old. Flow cytometry analysis the number of CD4+ IL-17A+ cells (a) and CD4+IFN-γ+ cells (b) in the spleen. RT-qPCR analysis of the Th17 relative cytokines mRNA expression from spleen in lupus nephritis mice, including IL-17A (c)、IL-17F (d). The quantitative real-time PCR was repeated 3 times with consistent results. Data are presented as mean ± SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

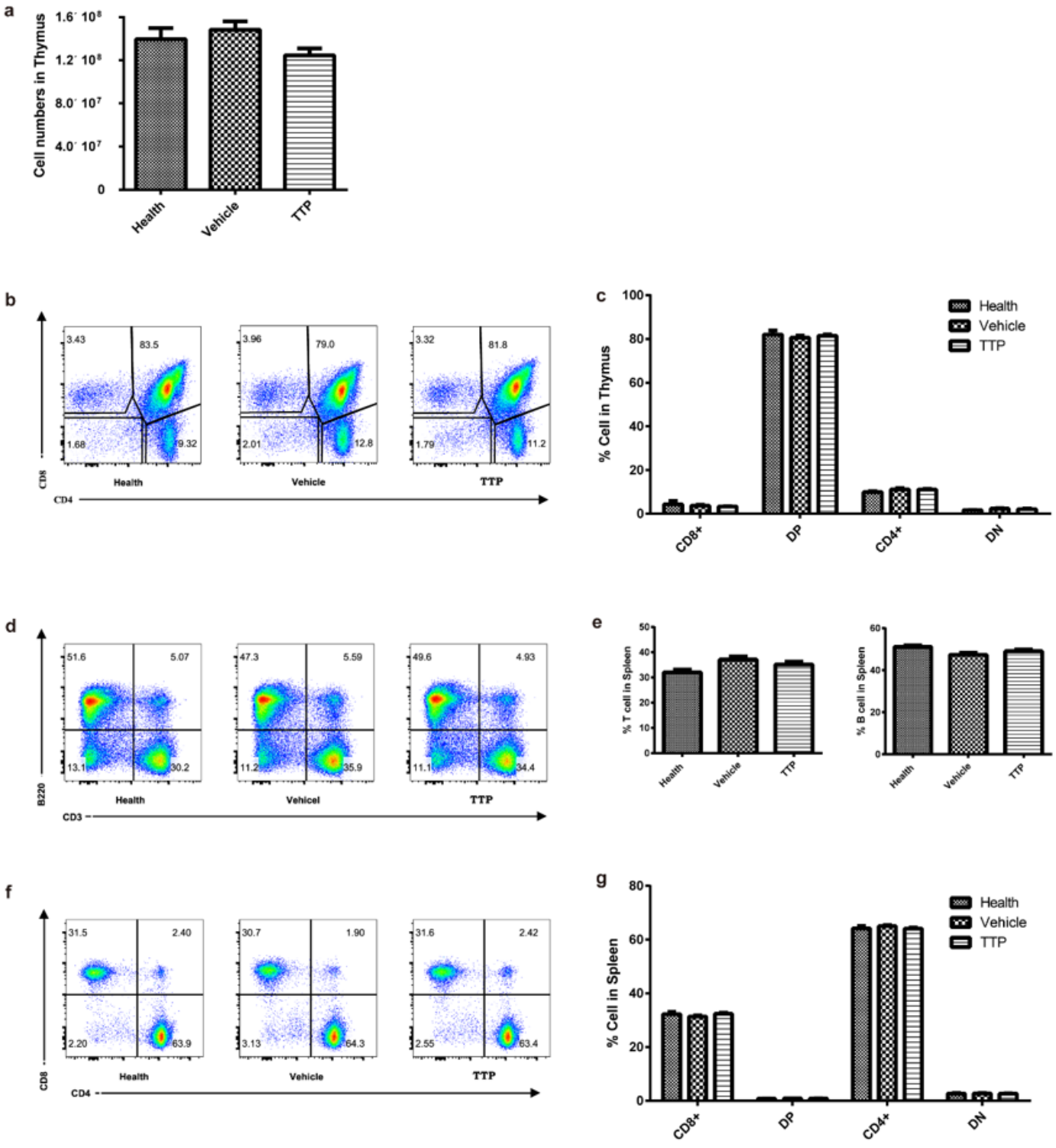

Figure 4

TTP has not affected thymic development in mice. The spleen and thymus were collected from 8 weeks old BALB/C mice which were treated with TTP or vehicle for 4 weeks. The numbers of CD4 T cell, CD8 T cell and total cell in the thymus for 4 weeks (a). The frequency of CD4 T cell and CD8 T cell in the thymus (b, c), the representative flow analysis result (b) and the statistical result (c). The proportion of T and B cells in the spleen for 4 weeks (d, e), the representative flow analysis result (d) and the statistical result

(e). The proportion of CD4 T cell and CD8 T cell in the spleen (f, g), the representative flow analysis result (f) and the statistical result (g). Results were expressed at the mean ± SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

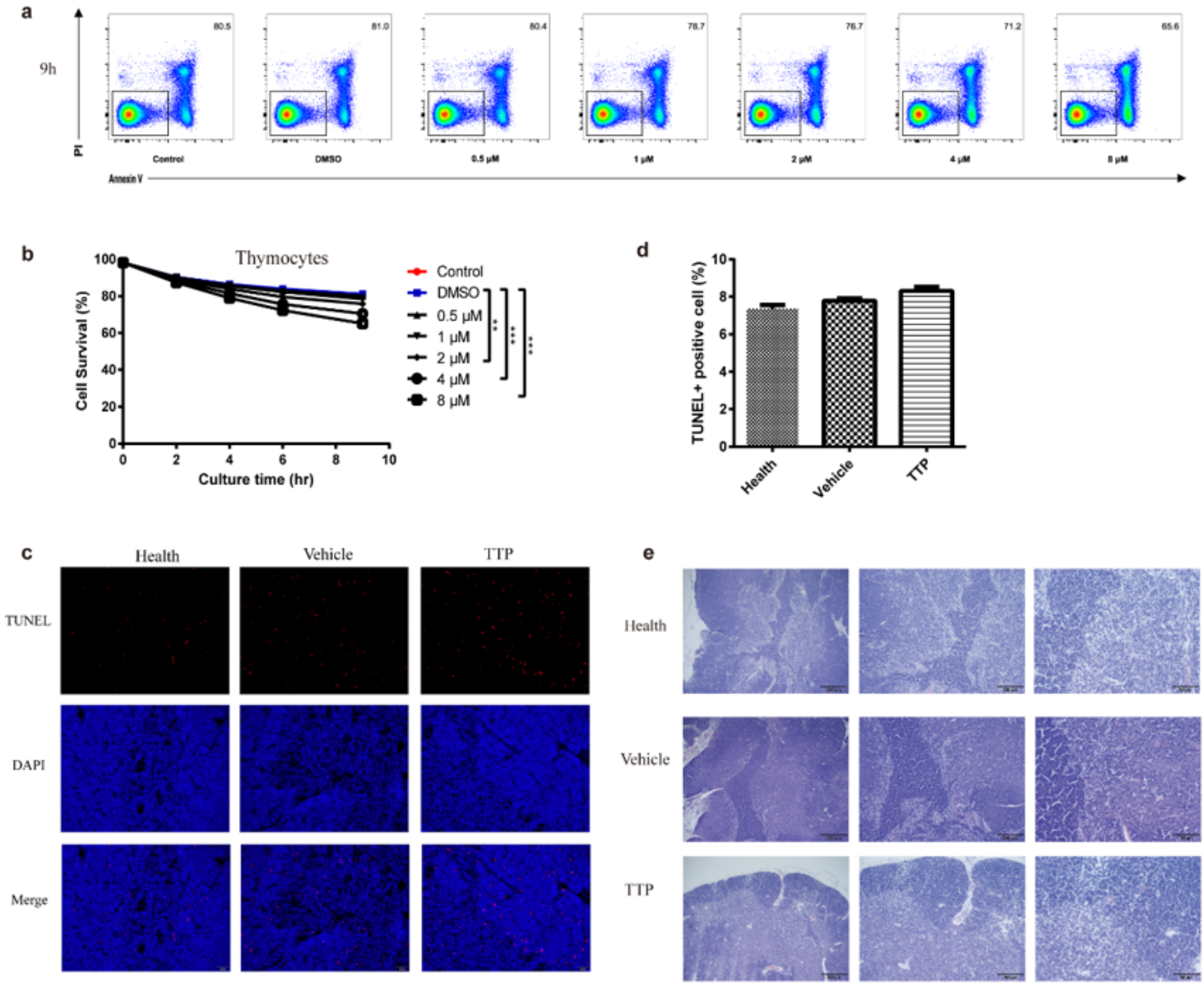

Figure 5

Analysis the apoptosis thymocytes and thymic alteration in the mice under treatment with TTP. The thymus was collected from 8-weeks C57Bl/J mice (a-d). The thymocytes were cultured with different concentrations of TTP for 1-9 h in vitro, stained for the apoptosis marker annexin V and PI, and analyzed by flow cytometry. Representative flow cytometry results were showed (a), thymocytes were cultured with TTP for 9h. And the statistical data were showed (b). The thymus was collected from 8 weeks old BALB/C mice which were treated with TTP or vehicle from 4 to 8 weeks old. The apoptosis of thymocytes was detected in health, vehicle and TTP groups by TUNEL staining (c), and the frequency of TUNEL+ cells in different groups was showed (d). 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=50μm. N=5. The

thymuses were collected from 12 weeks old BALB/C mice which were treated with TTP or vehicle from 4 to 12 weeks old (e). H&E stained sections of thymus form health (e, Top), vehicle-treated (e, Middle) and TTP-treated (e, Bottom). The scale bars were 200μm (left), 100μm (middle), 50μm (right). N=6-7. Results were expressed at the mean ± SEM. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

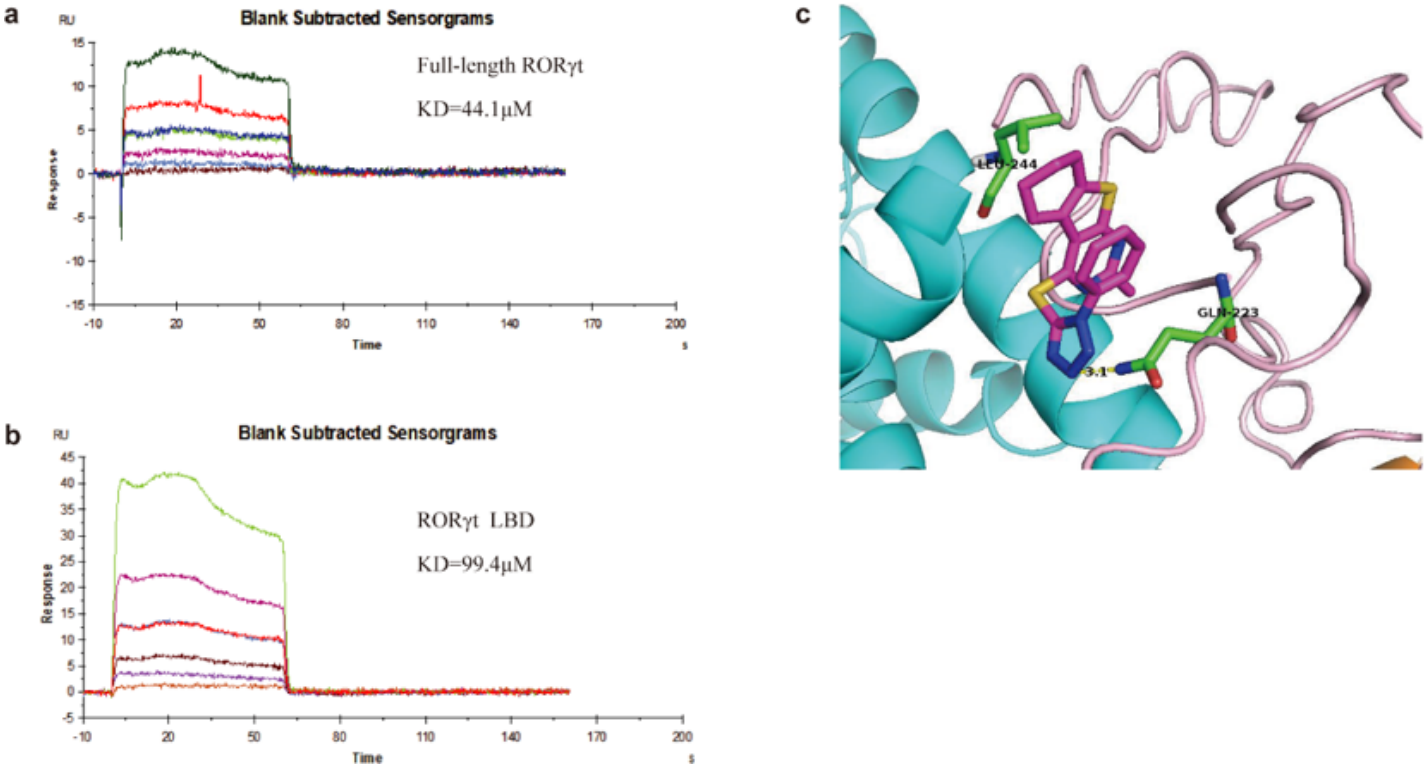

Figure 6

The binding mechanism of TTP with RORγt. Surface plasmon resonance experiment showed TTP (60, 30, 15, 7.5, 3.75, 1.875μM) bound with full-length RORγt protein (a) and RORγt LBD protein (b). The affinity of TTP bound with full-length RORγt protein and RORγt LBD protein were 44.1μM and 99.4μM, respectively. TTP (magenta) may form electrostatic interaction and hydrophobic interaction with Gln-223 and Leu-244 of RORγt respectively (c).