Therapeutic Effects of Triptolide on the Balance of Th17/Treg Cells via AKT/mTOR/p70S6k Signaling Pathway in Lupus-like Mice

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Research Article

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Abstract

T cell subsets play a critical role in immune regulation. T helper 17 (Th17) cells induce tissue inflammation and autoimmune response while regulatory T (Treg) cells mediate autoimmune tolerance and inhibit autoimmune response. Recent studies have shown that the important factors for the pathogenesis and disease activity of systemic lupus erythematosus (SLE), a chronic inflammatory autoimmune disease, are an increased number of Th17 cells and a decreased number or reduced functions of Treg cells. Triptolide is the main active ingredient of Tripterygium wilfordii Hook F (TWHF). It not only has anti-inflammatory, antiproliferative, immune modulation, and proapoptotic activity effects, but is also effective in treating SLE. However, the underlying mechanism of how triptolide works is still unclear. For the purpose of evaluating the impact of triptolide on the induction of Th17 and Treg cells, we conducted relevant experiments with respect to the number and activities of Th17 and Treg cells in *vivo* and in *vitro*. In *vivo*, intragastrically administered triptolide effectively ameliorated the clinical and histological symptoms in lupus-like mice, increased the number of induced Treg cells and reduced the number of Th17 cells in the spleen and their secretion. In *vitro*, triptolide is able to promote the differentiation of Treg cells and inhibit Th17 formation by inhibiting the AKT/mTOR/p70S6K pathway. Therefore, we posit that triptolide can help to deter the development of inflammation and ultimately treat SLE through regulation of the Th17 and Treg cells balance.

1. Introduction

Systemic lupus erythematosus (SLE) is a chronic inflammatory autoimmune disease, in which failure of endogenous mechanisms of immune tolerance often causes a range of immunoregulatory abnormalities that, in turn, lead to injuries of tissues and organs [1]. T cells play an important role in the pathogenesis of autoimmune diseases, including SLE[1–2]. T helper 17 (Th17) and Regulatory T (Treg) cells are distinct subsets of initial CD4 + T cells induced by different cytokines. Th17 cells could induce tissue inflammation and autoimmune response, while Treg cells mediate autoimmune tolerance and inhibit the autoimmune response. The high ratio of Th17 to Treg plays a key role in the pathogenesis and disease activity of SLE [3–4]. Tregs, expressing the master transcription factor Foxp3, are crucial for peripheral self-tolerance maintenance and autoimmunity limitations [5]. It does so by suppressing pathogenic immune cells, for instance, it would suppress autoreactive T cells that are over-activating or expanding [6–7]. RORγt is a transcription factor of the RAR-related orphan nuclear receptor family and is expressed in Th17 cells [8]. Therefore, RORγt is vital for the development and functioning of Th17.

The serine/threonine kinase mammalian target of rapamycin (mTOR) signaling pathway is involved in regulating cell metabolism and proliferation [9]. Akt, an important target for regulating protein translation, can activate the mTOR pathway via Serine 2448 (Ser2448) phosphorylation, thereby activating the protein p7056K to enhance transcription and translation of mRNAs and control cell growth [10]. Recent gene ablation studies have further demonstrated that the mTOR signaling pathway plays a critical role in T cell differentiation [9]. Absence of mTOR expression is proved to inhibit differentiation of Th17 cells, while increasing the induction of Treg cells [11]. Further study has found that mTOR is necessary for the

differentiation of Th17 cells [12], and mTOR signal differentiates Th17 cells mainly by activating STAT3 and RORγ T pathways and inhibits the differentiation of Treg cells by inhibiting Foxp3 expression [13–15]. mTOR signal pathway plays an important role in regulating Th17/Treg immune imbalance [16]. The increased expression of mTOR in SLE patients suggests that mTOR is critical for promoting autoimmune reactions by causing an imbalance of Th17/Treg-mediated immune homeostasis [17–18]. Indeed, mTOR signaling pathway is involved in the pathogenesis of SLE by regulating immune cell differentiation and inflammatory cytokine secretion [19]. Since protein translation is important in the synthesis of cell components and effector molecules in T cells and other cell types, inhibitors of the mTOR pathway, such as rapamycin, have been shown to block the activation of T cells [20]. In addition, rapamycin can reduce glomerular immunoglobulin deposition in lupus mice and effectively prevent the development of lupus [21]. Moreover, rapamycin can inhibit the activity of mTOR complex 1 (mTORC1) by increasing the activity of Foxp3 and promoting the differentiation of initial CD4 + T cells into Treg through signal transduction [22]. In human trials, rapamycin can help restore the Th17/Treg balance and decrease disease activity in patients with SLE [23]. As such, not only does mTOR activation play a key role in regulating T cell dysfunctions in systemic autoimmune diseases, it also shows great potential of being a therapeutic target in treating SLE.

Triptolide, a diterpene triepoxide extract from Tripterygium wilfordii Hook F (TWHF), has been shown to have immunosuppressive effects and has been proven to have certain pharmacological activities, the effects of which include anti-inflammation, immune modulation, antiproliferative and proapoptotic activities [32–36]. Tao *et al.* discovered that triptolide with 15 weeks administration could increase the survival rate, reduce disease severity and decrease cytokine production in mice with lupus nephritis (LN) [37]. Lu-Yao Zhang *et al*. discovered that (5R)-5-hydroxytriptolide could ameliorate lupus nephritis in MRL/lpr mice by inhibiting immune cell infiltration [38]. Zhao *et al*. found that triptolide can ameliorate lupus by inducing miR-125a-5p, mediating Treg upregulation [39]. However, there are relatively few studies on whether triptolide treats SLE by inhibiting mTOR signaling pathway and if so, the mechanisms of how it’s done.

Past studies have shown that triptolide can produce anti-inflammatory effects and ameliorate lupus by inhibiting the activity of effector T cells and inflammatory cytokines [39–40]. However, while Tregs are important in the inflammatory process of response, mTOR signaling pathway is highly activated in SLE T cells and plays a crucial role in promoting T cell differentiation. Nevertheless, the mechanism of triptolide in SLE remains unclear. Therefore, we wanted to explore triptolide’s effect on the regulation of the balance of Th17 and Treg cells in the SLE model by targetingthe mTOR pathway.

2. Materials And Methods

Animals. 6 to 8-week-old female BALB/c mice weighing 15–20g were purchased from the Experimental Animal Center of Shanghai, Chinese Academy of Sciences. All mice were maintained under specific pathogen free (SPF) conditions with a 12-hour light/dark cycle.The study was conducted in accordance with internationally accepted principles for the use and care of laboratory animals and was approved by

the Ethics Committee of Zhejiang University of Traditional Chinese Medicine (approval number: scxk – 2018-0006; Hangzhou, China).

Drugs and Reagents. Triptolide of 98% purity, as verified by HPLC (Sigma, St. Louis, MO, USA), was dissolved in dimethyl sulfoxide (DMSO) at a stock concentration of 20mg/ml. Naïve $CD4^+$ T cell Isolation Kit II was purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). LEAF™-purified anti-human CD3 and CD28 antibodies were purchased from BioLegend (San Diego, CA). FITC-conjugated anti-mouse CD4, PE-conjugated anti-mouse CD25, PE-conjugated anti-mouse FOXP3 antibodies and PE-conjugated anti-mouse IL-17A antibodies were purchased from Invitrogen eBioscience (Avenue Waltham, MA, USA). Anti-AKT, anti-pAKT (Ser473), anti-mTOR, anti-pmTOR (Ser2448), anti-P70S6K, and anti-pP70S6K (Thr389) antibodies were purchased from Cell Signaling Technologies (Danvers, MA, USA). Prednisolone acetate tablets were purchased from Shanghai Xinyi Pharmaceutical Co., Ltd., and Imiquimod (IMQ) (5%) cream from Hubei Keyi Pharmaceutical Co., Ltd.

Grouping and Treatment. Eight mice were selected at random as the control group. The remaining 48 mice were the triptolide group, induced with IMQ to be lupus-like autoimmune disease models and then treated with triptolide. For the triptolide group mice, 1.25mg of 5% IMQ cream were applied topically to the skin on the right ears of the mice 3 times per week for 8 weeks. Two weeks later after modeling, the 48 mice were randomized into six groups (8 mice per group): model group, low-dose treatment group (triptolide 0.1mg/kg/d), mid-dose treatment group (triptolide 0.2mg/kg/d), high-dose treatment group (triptolide 0.3mg/kg/d), prednisone treatment group (prednisone 5mg/kg/d), and rapamycin treatment group (rapamycin 100mg/kg/d). The model group and control group were treated with 0.9% saline 0.4ml/d by oral administration. Animals were sacrificed after 6 weeks of administration.

Measurement of urinary protein and serum chemistries. After model establishment, naïve contents of urinary protein were measured using random urine samples at 8–10 a.m. of the 0, 3rd and 6th week. All mice were free to access water but forbidden to access food during the period of sample collection. Urinary protein was evaluated by Coomassie brilliant blue staining. Blood samples were collected after the last administration and then the mice were sacrificed. The spleens were weighed. The kidneys were removed following sacrifice and fixed in 5% buffered formalin. Serum was isolated by centrifugation for 15 min at 4°C (3500 rpm). The blood samples of mice were collected for the analysis of liver function and kidney function. The levels of TGF-β, IL-17A, anti-dsDNA antibody (IgG) and smith antibody concentration in each group were evaluated by enzyme-linked immunosorbent assay (ELISA).

Histopathologic assessment. The renal tissues were dehydrated through a series of graded alcohol and embedded in paraffin. Paraffin-embedded kidney sections (3μm-thick) were stained with hematoxylin-eosin (H&E) and periodic acid-schiff stain (PAS). Glomerular lesions were graded semiquantitatively on a scale of 0–2, including mesangioproliferation, endocapillary proliferation, mesangial matrix expansion and segmental sclerosis (0 = 10%; 1 = 10–50%; 2 ≥ 50% of the glomeruli examined). Global glomerular lesion scores were evaluated under 400× light microscope fields 50 glomeruli at least and renal tubular damage was evaluated under 400× light microscope fields.

Immunofluorescence. Immunofluorescence staining was carried out on frozen sections embedded in cryo-embedding media (OCT compound). Renal tissues were sectioned at 5 μm using freezing microtome, stained with 1:100 diluted rabbit anti-mouse IgG antibody (Proteintech Group, Wuhan, China) at 4°C overnight and incubated with FITC or CyTM3-conjugated goat anti-rabbit IgG (Proteintech Group, Wuhan, China). These sections finally were mounted by DAPI-Fluoromount-G clear mounting agents and examined under an inverted microscope with 8 micrographs per slice obtained randomly (magnification 400×). The fluorescence signal was quantified using image-processing software (Image J 1.47), with six sections per group. The data were presented as average density (per pixel) (average density = integrated density/area).

Cell isolation and sorting. Splenocyte were isolated from the spleen of female BALB/c mice at 8 weeks old. Mononuclear cells were prepared by Ficoll-Hypaque density gradient centrifugation after passing through a 70 μm pore mesh. Cells obtained from the gradient interface were washed twice in PBS, and immediately separated naïveive $CD4^+$ T Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany).

T cells culture and stimulation. The isolated $CD4^+CD25^-$T cells were cultured in 96-well at a concentration of $1x10^5$ cells/well. Cells were stimulated with 2 μg/ml anti-CD3 (clone 145-2C11; BD Biosciences) and 2 μg/ml anti-CD28 (clone 37.51; BD Biosciences). For Th17 cell induction, cells were cultured with 2.5 ng/ml transforming growth factor β-1 (TGF-β1) (R&D systems), 20 ng/ml interleukin 6 (IL-6) (R&D systems), 10 μg/ml anti-IL-4 (Pepro Tech) and 10 μg/ml anti-IFN-γ (Biolegend). For Treg cell induction, cells were cultured with TGF-β1 (2.5 ng/ml; R&D systems) and interleukin 2 (IL-2) (10 ng/ml; BD Biosciences). The culture medium consisted of RPMI 1640 (Gibco) supplemented with 10% fetal calf serum (Hyclone), 1 mM sodium pyruvate (Sigma-Aldrich), 50 μM β-mercaptoethanol (Sigma-Aldrich), 0.1 mM non-essential amino acids (Sigma-Aldrich), 100 U/ml penicillin, 2 mM L-glutamine, 100 mg/ml streptomycin and 10 mM Hepes. Cells were harvested after 48 hours for further analysis.

Cell viability measurements. Th17 and Treg cells were induced in 96-well plates at 1.0×10^5/well and then treated with vehicle, rapamycin or triptolide (0-80nM) for 72 hours. The cell viability was evaluated respectively by cell counting kit-8 (CCK-8) assay (KeyGEN, BioTECH, China).

In vitro grouping and administration. Purified $CD4^+CD25^-$T cells were cultured in 96-well plates and stimulated with anti-CD3/CD28 for 72 hours. The optimal concentrations of triptolide on Thl7 and Treg cells detected by CCK-8 experiment were 2.5nM, 5nM and 10nM, which were selected as the following experimental concentrations for Th17 and Treg cells. The rapamycin group (100 nM) was considered as positive control. Cultured cells were harvested after 5 days and analyzed by flow cytometry.

Enzyme-Linked Immunosorbent Assay. The levels of TGF-β1 secreted by iTreg cells and IL-17 by Th17 cells were evaluated in splenic supernatants using TGF-β1 and IL-17 mouse ELISA (Wuhan Huamei Bioengineering Co., Ltd).

Flow cytometry analysis. As for Th17 cell percentage analysis ($CD4^+IL17A^+$T cells/$CD4^+$T cells %), prepared mononuclear cells were first stimulated by 25ng/ml phorbol myristate acetate (PMA, Sigma-Aldrich) and 1 μg/ml ionomycin (Sigma-Aldrich) in the presence of 2 mmol/ml monensin (Sigma-Aldrich) for 4 hours. Next, mononuclear cells were stained with FITC-conjugated CD4 antibody at 4℃ in the dark for 30 minutes. Then, cells were fixed, permeabilized, and stained intracellularly with PE-conjugated IL-17A antibody (eBioscience company). For Treg cell percentage analysis ($CD4^+CD25^+Foxp3^+$T cells/$CD4^+$T cells %), mononuclear cells were stained with FITC-conjugated CD4 antibody for 30 minutes, and intracellularly stained with APC-conjugated CD25 antibody (eBioscience company) and PE-conjugated Foxp3 antibody (eBioscience company) respectively for 45 minutes. Flow cytometric analysis was performed on a FACScanto flow cytometer (BD Biosciences company, San Jose, CA, USA). Experiments were performed in triplicate. Isotype controls were used to correct nonspecific binding in all procedures.

Quantitative reverse transcription–polymerase chain reaction (PCR). RORγt and Foxp3 mRNA were determined by Reverse Transcription-Quantitative PCR (RT-qPCR). Total RNA was isolated from splenocytes using the MiniBEST Universal RNA Extraction kit (Takara Bio, Inc., Japan). RNA reverse transcription was carried out by using PrimeScript RT Master Mix (Takara Bio, Inc., Japan). The reactions were performed by CFX96 (Bio-Rad Laboratories, Inc., USA). The primer sequence of each target mRNA is as follows: 5’-ACGGCCCTGGTCT-CATCA-3’ and 5’-CCAAATGTATGCAGATGTCCAC-3’ for RORγt; 5'-AGTTCCTTCCCAGAGTTCTTCCA-3' and 5'-GCTCAGGTTGTGGCGGATG-3' for Foxp3; 5’-ACCGCACTCCCTCTCTCGTAT-3’ and 5’- TGGCGTGAGGGAGAGCATAG – 3’ for β-actin. $2^{-\Delta\Delta Cq}$ method was used to quantify the relative mRNA expression.

Western blotting Total protein was extracted from the cultured cells and blotted onto PVDF membranes, which were then blocked in TBST containing 5% skimmed milk for 1 hour. The membranes were incubated with antibodies against AKT, p-AKT, mTOR, p-mTOR, P70S6K, p-P70S6K, and GAPDH (1:1000, all from CST, Beverly, MA) at 4℃ overnight. On the second day, the membranes were incubated with biotinylated goat anti-rabbit IgG or goat anti-mouse IgG (1:2000, CST, Beverly, MA) for 2 hours at room temperature. The signals were visualized by ECL Advance reagent (Millipore, Billerica, MA) and the specific protein bands were quantified using ImageLab software.

Statistical analysis

All data were expressed as Mean ± SD. Differences between groups were analyzed by independent-samples t-tests and one-way analysis of variance using SPSS 17.0 software. $P < 0.05$ was considered statistially signiicant.

3. Results

3.1 Triptolide ameliorates clinical symptoms of lupus-like mice

After the mice were sacrificed, spleen index (ratio of spleen weight to body weight) was reduced significantly in triptolide groups of each dose. The spleen index in the rapamycin and prednisone groups was also lower compared with model group (Fig. 1A-B). To assess the development of nephritis in lupus-like mice, proteinuria was monitored every 3 weeks. As shown in Fig. 1C, 8-week-old lupus-like mice (before treatment) had higher level of proteinuria. The mean level of urinary protein was not significantly different at 8 week-old age between the 6 groups (Fig. 1C). With aging, proteinuria in triptolide-treated mice was decreasing progressively following the increase of triptolide concentration compared with the model group. The onset of severe proteinuria was significantly delayed in treated mice. Meanwhile, levels of serum creatinine (Scr) and urea nitrogen (Bun) after administration (Fig. 1D-E) were detected for the evaluation of kidney function. The model group had severe proteinuria with an increase in Scr levels (Fig. 1D). In contrast, mice treated with triptolide (mid-dose), rapamycin and prednisone maintained lower Scr level 6 weeks after the treatment. Furthermore, serum anti-double-stranded DNA (anti-ds-DNA) Ab and Smith levels in the model group were higher than those in the control group. Treatment with triptolide (mid- and high-doses), prednisone or rapamycin could significantly lower the level of circulating anti-ds-DNA and Smith antibody (Fig. 1F-G). The levels of IL-17 in the mid-dose and high-dose triptolide group were significantly lower than those in the model group ($p < 0.05$) (Fig. 1H). The levels of TGF-β in the serum of each group treated with triptolide were higher than those in the model group($p < 0.05$) (Fig. 1I).

3.2 Triptolide Ameliorates The Histological Lesions Of Kidneys

Histopathologic assessment of the kidney in the model group showed enlarged glomeruli, an increase in the mesangial matrix, mild peritubular mononuclear cell infiltrates, and a significant increase in the renal histopathologic score (Fig. 2A-B,D). In contrast, glomerular lesions in mice treated with triptolide were milder in the mesangial cell proliferation and less severe in the interstitial inflammation. The renal pathological scores of all treatment groups were decreased in varying degrees, especially in the high-dose triptolide group ($p < 0.05$). Excessive deposition of immune complexes in glomeruli plays a key role in the pathogenesis of lupus nephritis (LN). Immunological staining of the kidneys in the model group showed severe granular deposition of IgG in glomeruli, while mice with triptolide, rapamycin or prednisone treatment had significantly decreased deposition of IgG in the glomeruli (Fig. 2C)

3.3 Triptolide influences the balance between Th17 and Treg cells

To analyze the effect of triptolide on the balance between Th17 and Treg cells, $CD4^+IL\text{-}17^+$Th17 and $CD4^+CD25^+Foxp3^+$ Tregs were isolated from the spleen and numerated by fluorescence-activated cell sorting (FACS). As shown in Fig. 4, triptolide treatment significantly increased the frequency of

$CD4^+CD25^+Foxp3^+$ Tregs in spleens and reduced the frequency of $CD4^+IL\text{-}17^+$Th17, suggesting that triptolide could regulate Th17/Treg immune imbalance. To verify the role of triptolide in Treg cell differentiation, we stimulated the purified $CD4^+CD25^-$ T cells in *vitro* with TGF-β, IL-6 and IL-2 in the presence or absence of triptolide. The frequency of $IL\text{-}17^+$ T cells was markedly reduced in cultured cells with exposure to triptolide (Fig. 5C). Meanwhile, the frequency of $Foxp3^+$ T cells was significantly increased when co-cultured with triptolide (Fig. 5D). These results are consistent with previous reports showing that triptolide could alter the imbalance of Th17/Treg cells [34]. Subsequently, we analyzed the expression of RORγt mRNA, a core transcription factor regulating Th17 cells. Our results showed RORγt mRNA expression could be down-regulated by triptolide treatment (Fig. 7B). Previous studies demonstrated that Treg cells require Foxp3 to activate and maintain Foxp3 expression [5], while our results showed that triptolide treated cells had up-regulated expression of foxp3 compared with the control group (Fig. 7A). Moreover, triptolide treatment increased the expression of Foxp3 mRNA, and decreased the expression of RORγt mRNA *in vivo* (Fig. 3). Collectively, these data indicate that triptolide could inhibit the Th17 cell differentiation process and promote the Treg cell differentiation.

3.4 Triptolide Affects The Secretion Of Inflammatory Cytokines

To evaluate the effect of triptolide on the levels of inflammatory cytokines *in vitro*, TGF-β was detected in the supernatant of the Treg cells while IL-17 was detected in the supernatant of the Th17 cells. Triptolide at 2.5nM, 5nM, 10nM could significantly promote the secretion of TGF-β in Treg cells and decrease the levels of IL-17 in Th17 cells on day 7 (Fig. 6).

3.5 Triptolide affects the expression of phosphorylated Akt, mTOR and p70S6K.

To analyze the role of triptolide in modulating AKT/mTOR/p70S6K signaling, which is considered as the main pathway regulating Th17 and Treg cell differentiation, the expression of AKT, mTOR, p70S6K as well as the phosphorylated protein were evaluated respectively in the Th17 and Treg differentiation system. As a result, triptolide at 2.5nM, 5nM, 10nM and rapamycin inhibited the phosphorylation of AKT, mTOR, p70S6K both in Th17 and Treg cells (Fig. 8). Therefore, it suggests that triptolide promotes Treg cell differentiation and inhibits Th17 cell differentiation by inhibiting AKT/mTOR/p70S6K signaling pathway.

4. Discussion

SLE is a heterogeneous chronic autoimmune disorder that causes inflammation and is characterized by the progressive involvement of multiple organs and systems in the body. Therefore, it is very important to find effective drugs to treat SLE. In this study, TLR-7 agonist acting on the epidermis was used to induce lupus erythematosus, the symptoms of which are similar to SLE [35].

It is well-known that numerous herbal extracts and small molecular ingredients attained from certain herbs have been effective when used to treat autoimmune diseases. Examples include artemisinin, tripterygium glycosides and total glucosides of paeony [36–38]. Previous studies have established that triptolide has immunomodulatory properties, including anti-inflammatory, antioxidant, immunomodulatory, antiproliferative and proapoptotic activities [24–26]. Triptolide has been found to be effective in treating autoimmune diseases such as SLE, rheumatoid arthritis, vasculitis and psoriasis [31–32, 38–39]. It is the most effective monomer with the strongest immunosuppressive and anti-inflammatory activity among a variety of Tripterygium wilfordii extracts. It can regulate the balance of Th17/Treg [31, 40] and plays an important role in immune response. Moreover, studies have shown that triptolide can reduce levels of inflammatory cytokines and prevent excessive activation of effector $CD4^+$T cells [32, 33]. Studies on animals show that triptolide can inhibit the production of inflammatory mediators and it seemed that renal function in lupus mice improved drastically [31, 41].

Triptolide has anti-inflammatory and immunomodulatory effects and is an herb extract that shows great potential when used to treat SLE. To investigate the protective effects of triptolide, we studied its effects on immunopathology and renal dysfunction. Using triptolide, renal function showed improvement, proteinuria weakened and renal pathological damage, inclusive of the deposition of IgG, attenuated. The various models of kidney injuries (e.g., chronic nephritis [42, 43], acute renal injury [44] and glomerulosclerosis [45, 46]) have also disclosed the benefits of triptolide with respect to renal functions. This indicates that triptolide protects renal function through various mechanisms. As with many autoimmune diseases, a notable increase of serum autoantibodies is also seen in SLE, which led us to investigate whether triptolide has an impact on the regulation of autoantibody responses in lupus-like mice. We found that anti-dsDNA Ab and Smith Ab in the serum significantly reduced, illustrating that triptolide can suppress humoral immunity. Overall, the data gathered from this study shows that through regulating cellular and humoral immune responses in lupus-like mice, triptolide can have anti-inflammatory effects.

As important cells of the immune system, Th17 and Treg are essential in regulating both the immune homeostasis and tolerance to self-antigens. In this study, we found that triptolide facilitated the secretion of cytokines TGF-β1 and inhibited simultaneously the secretion of cytokine of IL-17 in serum. As part of the study, we further explored the anti-inflammatory and immunomodulatory properties of triptolide through the induction ofTh17 and Treg cell *in vitro*. We found that intragastric administration of triptolide increased the level of Tregs and decreased that of Th17 in the spleens of mice. In addition, triptolide also markedly increased the expression of Foxp3 mRNA in the spleen and decreased RORγt mRNA in the spleen. Therefore, it seems that triptolide's anti-inflammatory and immunomodulatory properties are achieved by promoting the differentiation of Tregs and inhibiting Th17 formation. This finding is evidence of a potential new approach for treating inflammatory autoimmune diseases such as SLE.

In terms of the *in vitro* part of the study, we isolated spleen naive $CD4^+$ T cells and successfully established differentiation system of Th17 and Treg cells which were applied with triptolide to examine its effects on T cell differentiation. As with previous studies, we found that triptolide had a positive effect

on the induction of Treg differentiation and inhibiting Th17 cell production in a dose-dependent manner. Cytokine production by the Treg and Th17 cells were quantified to facilitate the study and detection of the cellular function. In line with our expectations, in supernatants from triptolide treated Treg cultures, we saw a dose-dependent increase in TGF-β levels and decrease in IL-17 levels from Th17 cell cultures. In addition, triptolide was shown to enhance the expression level of Foxp3 in Tregs while decrease RORγt expression in Th17 cells. Therefore, we suggest that triptolide's anti-inflammatory and immunomodulatory qualities are achieved through regulating the differentiation of Th17 and Tregs.

Previous studies have shown that the PI3K/AKT/mTOR signaling pathway plays a vital role in T cell growth, migration, proliferation, and metabolism [47], and phosphorylation of Akt-mTOR can promote Th17 differentiation [48] and inhibit Treg differentiation [49]. However, the contrary is observed when PI3K/AKT/mTOR signaling is suppressed. In this study, it was found that the proportion of Treg was enhanced and the Th17 decreased. Meanwhile, the inhibition of Akt/mTOR/p70S6K signaling pathways after triptolide treatment was applied suggests that triptolide can have a positive effect in the Treg/Th17 balance in lupus-like mice. Further study illustrated a negative correlation between AKT/mTOR/p70S6K expression and Treg frequency and a positive correlation in terms of Th17 frequency. The findings indicate that triptolide treatment for lupus could be related to the increased expression of AKT/mTOR/p70S6K signaling pathways and Treg/Th17 proportion. In the present study, triptolide markedly inhibited AKT, mTOR and p70S6K phosphorylation, leading us to believe that it is through the inhibition of PI3K/AKT/mTOR signaling that triptolide is able to regulate the Treg andTh17cells differentiation in peripheral tissues.

All in all, we have investigated and found evidence for why triptolide has therapeutic effects on lupus-like mice and clarified the cellular and molecular mechanisms. We found that triptolide was able to treat kidney injury and dysfunction in lupus-like mice by mediating Th17/Treg immune imbalance. Further, our study showed that triptolide's regulation of T cell immune balance is in affiliation with the downregulation of the mTOR/p70S6K pathway. As a result, the combination of our findings indicates that triptolide is a novel immunosuppressant that has the ability to inhibit mTOR-signaling.

5. Conclusion

Our study showed that triptolide can effectively improve the clinical manifestations and pathological changes in lupus-like mice, inhibit the formation of Th17 cells and promote the differentiation of Treg, which helps to regulate the balance of Th17 and Treg, which in turn, has anti-inflammatory and immune-regulation effects. Akt/mTOR/p70S6K signaling pathway is one of the mechanisms that regulate the balance of Th17/Treg, suggesting that triptolide could be a therapeutic candidate for the treatment of lupus-like autoimmune disease. Results from our study also highlighted that, by regulating the balance of Th17/Treg, triptolide can inhibit the development of inflammation and treat inflammatory autoimmun diseases such as SLE.

Declarations

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Authors' contribution

Qice Sun and Yan Liu are contributed equally to this paper, they were involved in the conception, design, the main part of the experiment and write-up of the manuscript. Dingqi Lu, Lina Ji, Weijie Wang and Xinchang Wang contributed to data acquisition and analysis, write-up of the manuscript. Yongsheng Fan and Guanqun Xie contributed to critical review of manuscript for important intellectual content and final approval of the mauscript.

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Available of data and materials

The data that support the findings of this study are available on request from the corresponding author. The data are not publicy available due to privacy.

Competing interests

The authors declare that they have no competinginterests.

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Figures

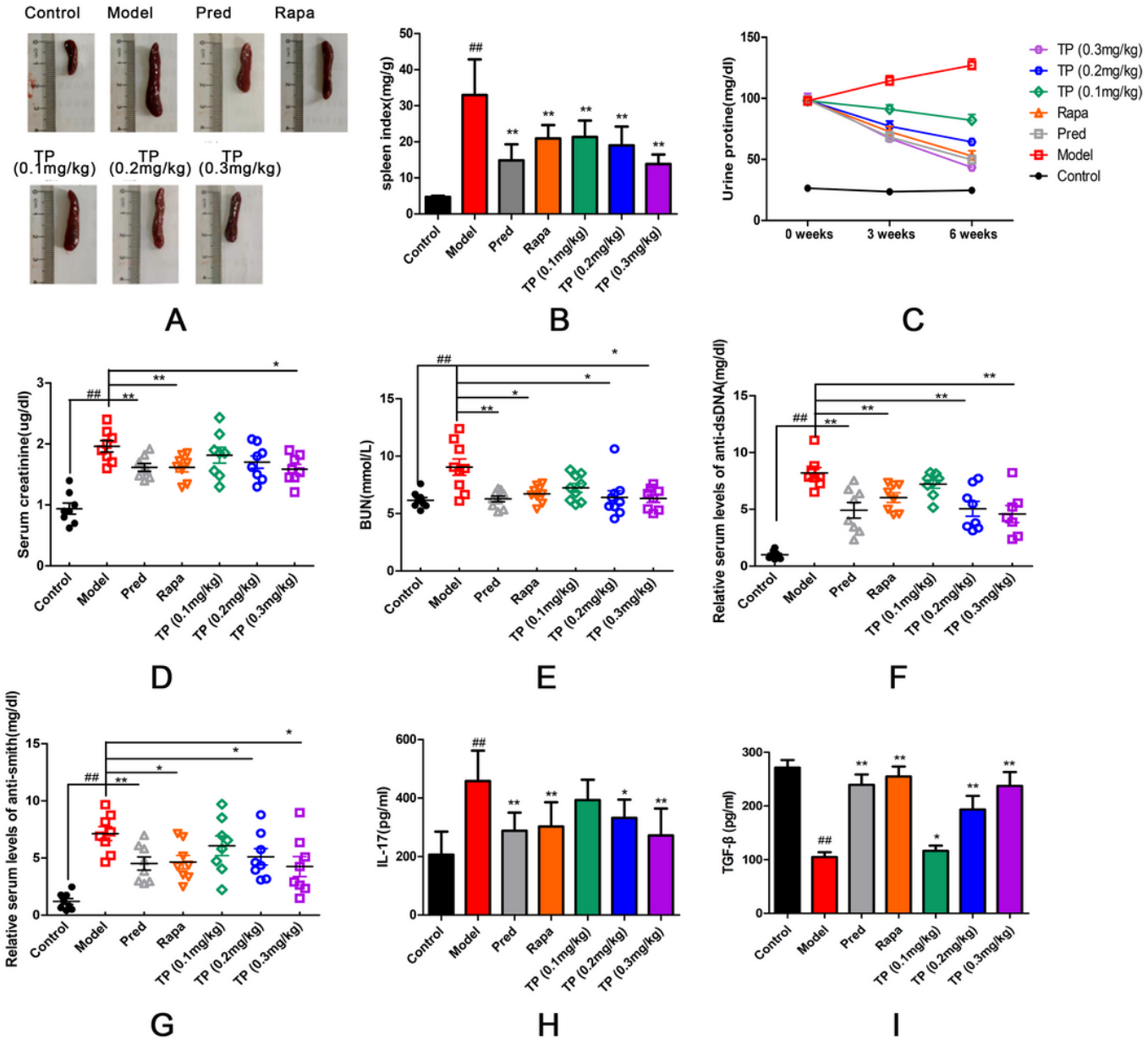

Figure 1

The effect of triptolide on spleen index, proteinuria, renal function and serum anti-dsDNA and Smith levels in lupus mice. A.B.The spleen size and spleen index (spleen weight/body weight) were observed at the end of the study. C.Random urine was collected every 3 weeks to observe the effect of triptolide on renal involvement of each group. D.E.F.G.The animals were sacrificed on the 6th week, and the serum creatinine (Scr), BUN, serum anti-dsDNA and anti-Smith antibodies of each group of mice were measured. H.I. IL-7 and TGF-βwere detected by ELISA. *P<0.05, **P<0.01, Pred means prednisone; Rapa means

rapamycin; TP meas triptolide. The data were shown as mean ± standard error of mean (n = 7 for each group). #P < 0.05, ##P < 0.01 vs control; *P < 005, **P < 0.01 vs model.

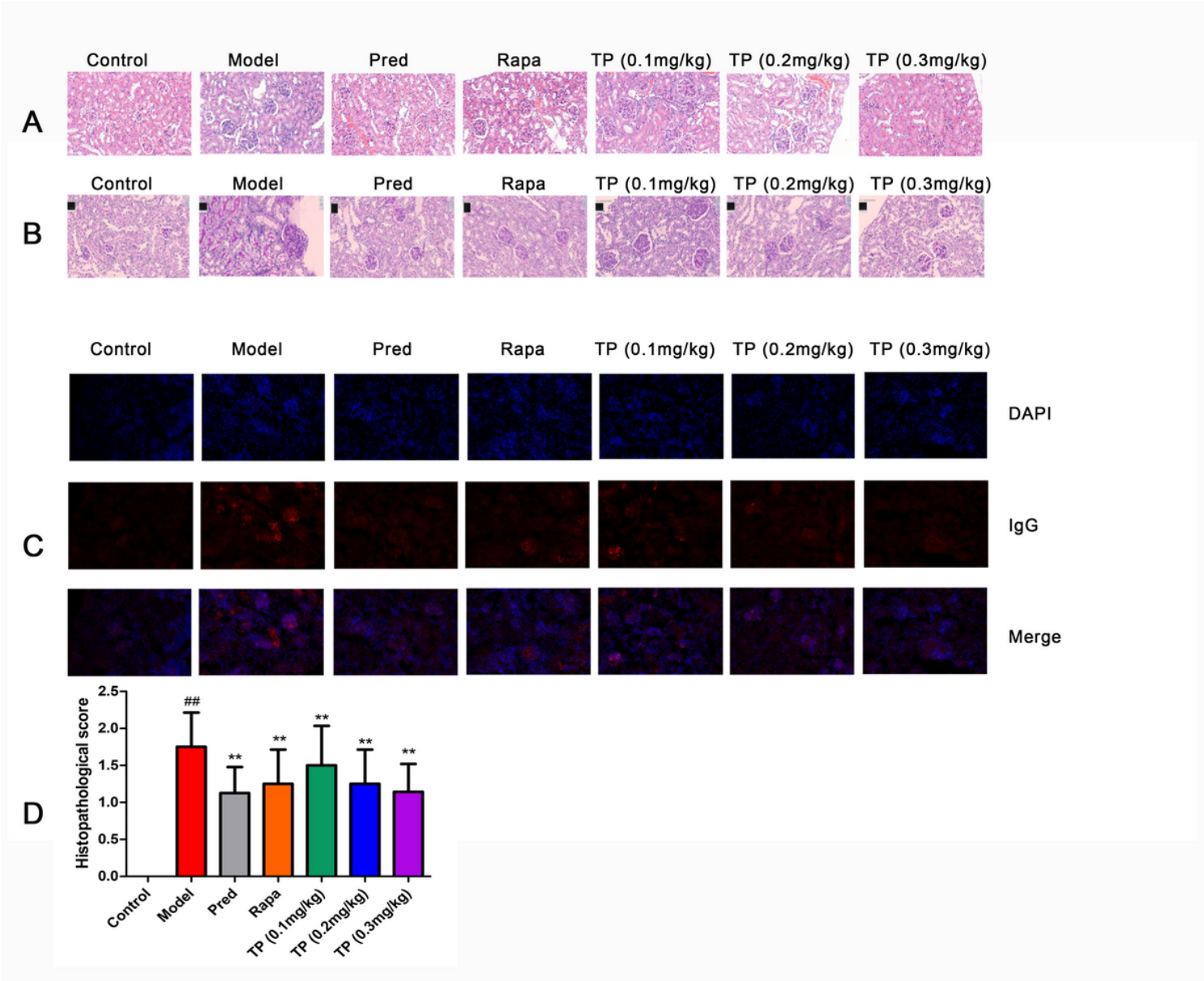

Figure 2

Effects of triptolide on kidney pathological changes and IgG deposition in imiquimod-induced lupus like mouse models. A, B H&E and PAS staining (magnification, x40). C Immunofluorescence staining of total glomerular IgG. ##P<0.01 compared to the control group, and *P<0.05 or **P<0.01 for all treatment groups. D Kidney pathological score.

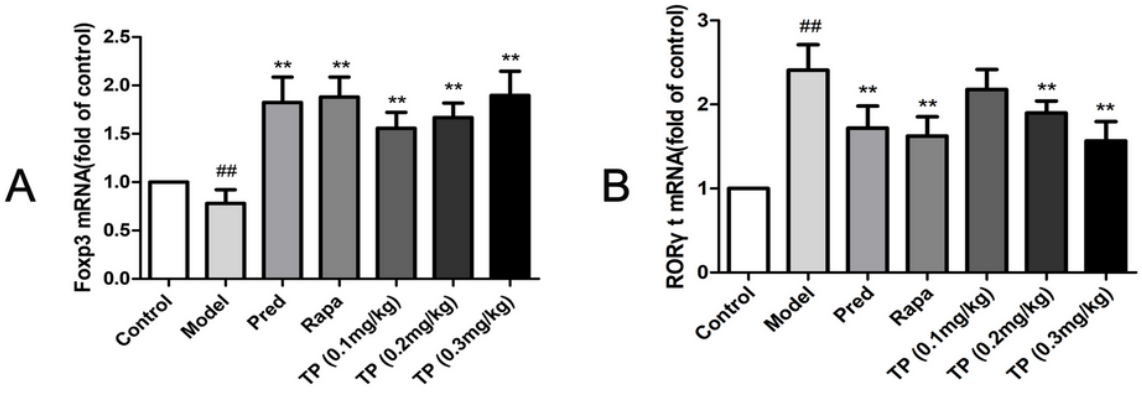

Figure 3

The effects of triptolide on expression of RORγt and Foxp3.A.B.The RT-qPCR method was used to detect the mRNA expression of transcription factors RORγt and Foxp3 in the splenic T lymphocytes of each group of mice. The histogram data is expressed as the mean ± SD of three independent experiments, ##P<0.01 compared to the control group, and *P<0.05 or **P<0.01 for all treatment groups.

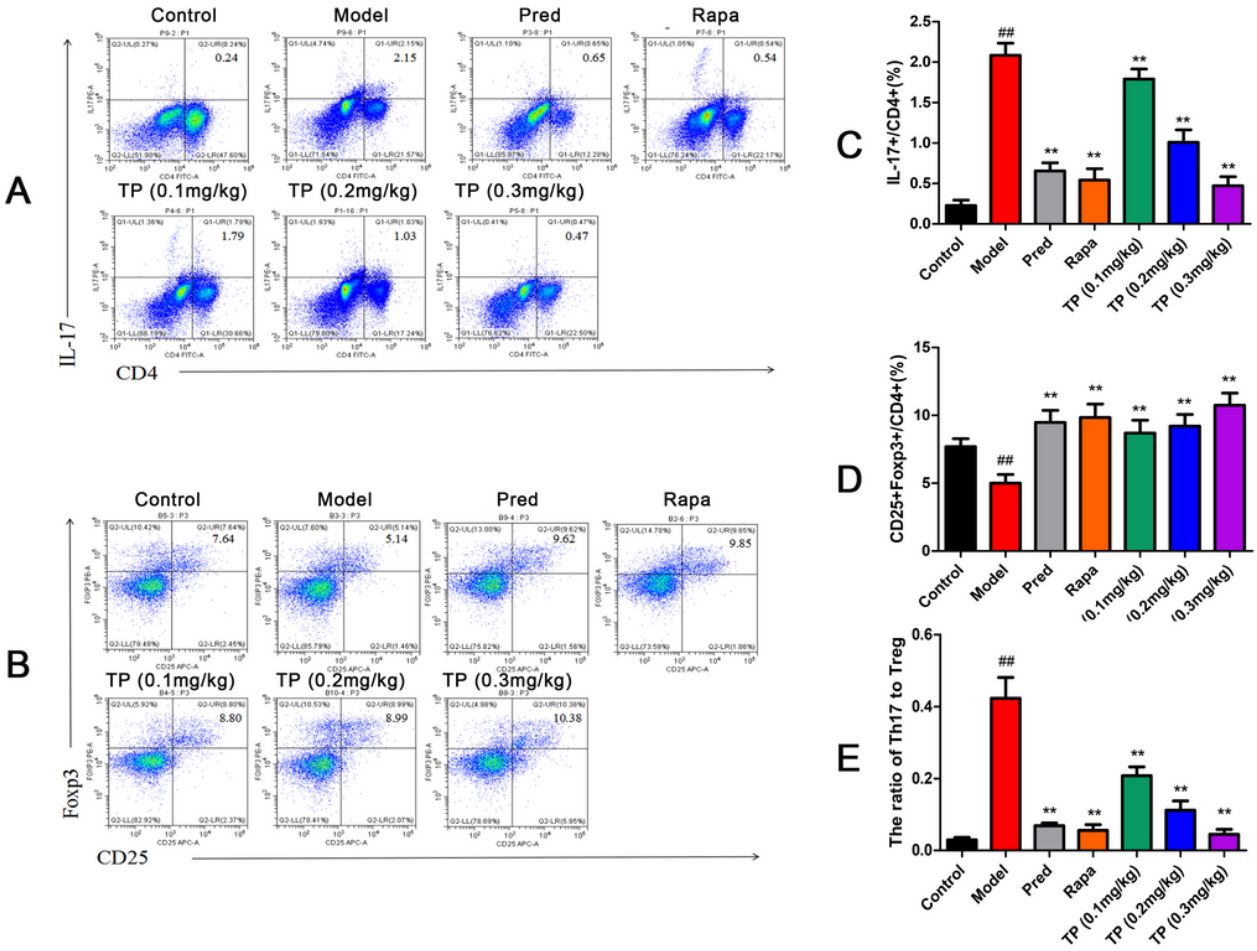

Figure 4

The effect of Triptolide on Th17 and Treg differentiation. A.B.C.D.CD4+ IL17+ was counted by FACS the frequency of Th17 and CD4+CD25+Foxp3+treg. E.The ratio of Th17 to Treg cells was calculated. The data are expressed as the mean±SD of three independent experiments (n=8). ## P<0.01 compared to the control group, and *P<0.05 or **P<0.01 for all treatment groups.

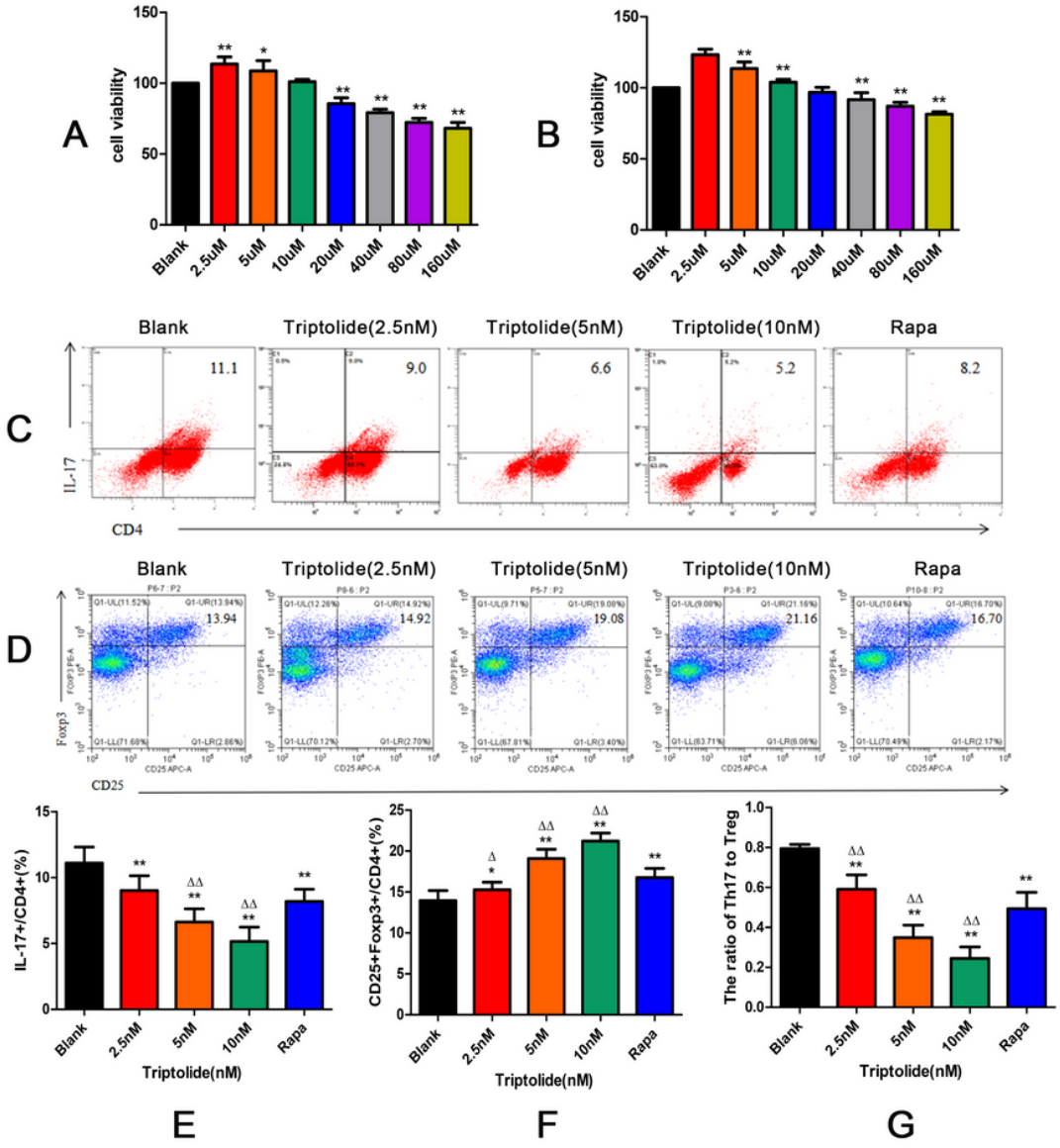

Figure 5

Effects of triptolide on the differentiation of Treg and Th17 cells induced in vitro. A.B.The viability of Th17 and Treg were evaluated when cultured with different concentrations of triptolide (0-50nM) by cell counting kit-8 (CCK-8) assay. C.D.E.F.The frequencies of $CD4^+$ $CD25^+$ $FoxP3^+$ Tregs and $CD4^+$ $IL\text{-}17^+$ Th17 were detected by FACS analyses seven days after the cell culture. G.The ratio of Th17 to Treg was calculated. The data are expressed as the mean±SD of three independent experiments (n=3). A-B:

**P<0.01 compared to the blank group; E-G: *P<0.05 or **P<0.01 compared to the blank group, ΔP<0.05 or ΔΔP<0.01 compared to the Rapa group.

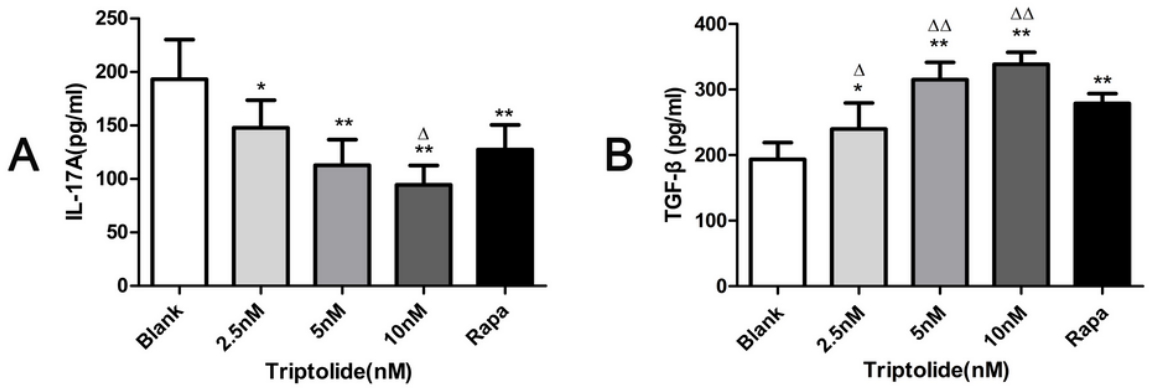

Figure 6

Triptolide affects the secretion of inflammatory cytokines. A.B. The levels of IL-17 in Th17 cells and TGF-β in Treg cells were detected. Data of column graphs are presented as the mean±SD of three independent experiments after one-way ANOVA with Tukey's posthoc test (n=3). *P<0.05 or **P<0.01 compared to the blank group, ΔP<0.05 or ΔΔP<0.01 compared to the Rapa group.

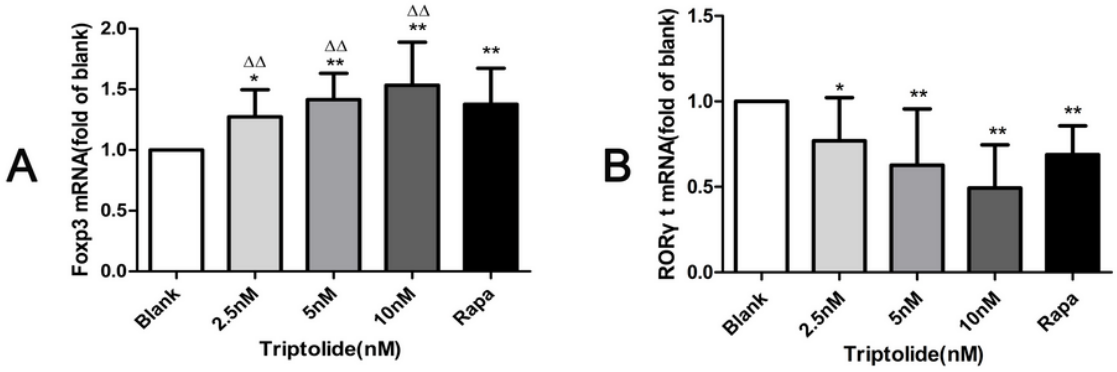

Figure 7

The effects of triptolide on the key inflammation gene expression of Foxp3 and RORγt in *vitro*. A.B.The inflammation associated genes expression of Foxp3 in Treg cells and RORγt in Treg cells measured by qRT-PCR. The data of column graphs are presented as the mean±SD of three independent experiments after one-way ANOVA with Tukey's posthoc test(n=3). **P*<0.05 or ***P*<0.01 compared to the blank group, Δ*P*<0.05 or ΔΔ*P*<0.01compared to the Rapa group.

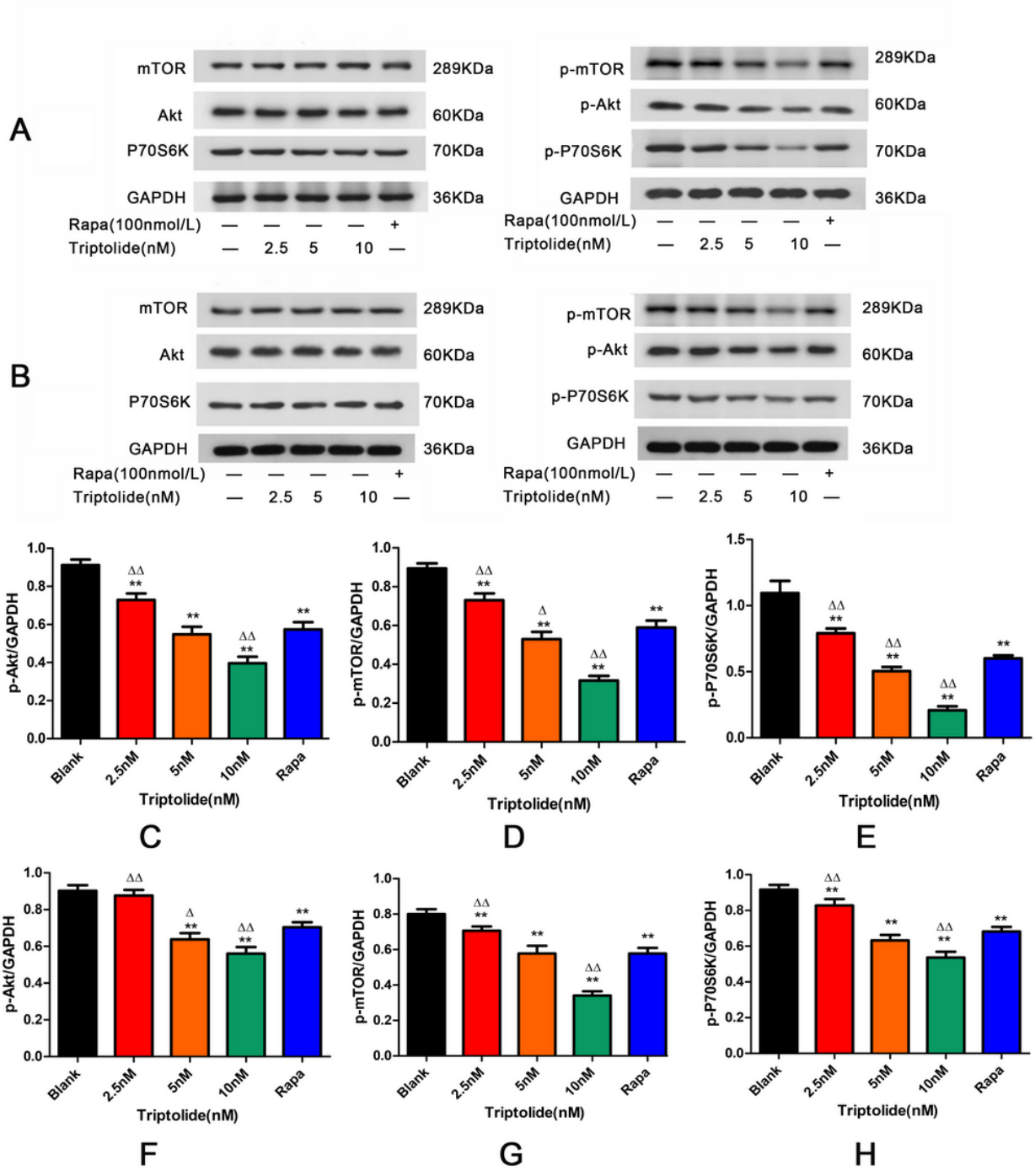

Figure 8

Triptolide inhibited the phosphorylation of AKT, mTOR and p70S6K. A.B.The protein expression of AKT, mTOR, and p70S6K as well as phosphorylated protein in the Th17 and Treg cells on the 7th day were detected by western blotting.C.D.E. The ratio of phosphorylated AKT to GAPDH, mTOR to GAPDH and p70S6K to GAPDH in Th17 cells. F.G.H.The ratio of phosphorylated AKT to GAPDH, mTOR to GAPDH and p70S6K to GAPDH in Treg cells. Data of column graphs are presented as the mean±SD of three

independent experiments after one-way ANOVA with Tukey's post hoc test(n=3). *P<0.05 or **P<0.01 compared to the blank group, ΔP<0.05 or ΔΔP<0.01 compared to the Rapa group.