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Triptolide ameliorates lupus via the induction of miR-125a-5p mediating Treg upregulation

Xia Zhao[a,b], Xiaojun Tang[c], Qing Yan[a], Hua Song[a], Zutong Li[c], Dandan Wang[c], Hongwei Chen[c], Lingyun Sun[a,*]

[a] Department of Rheumatology and Immunology, Drum Tower Clinical Medical College of Nanjing Medical University, 321 Zhongshan Road, Nanjing, Jiangsu 210008, China
[b] Department of Rheumatology, Affiliated Hospital of Nanjing University of Chinese Medicine, 155 Hanzhong Road, Nanjing, Jiangsu 210029, China
[c] Department of Rheumatology and Immunology, The Affiliated Drum Tower Hospital of Nanjing University Medical School, 321 Zhongshan Road, Nanjing, Jiangsu 210008, China

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ABSTRACT

Triptolide is a biologically active component of the Chinese antirheumatic herbal remedy Tripterygium wilfordii Hook F, which has been shown to be effective in treating murine lupus. However, its immunological mechanisms are poorly understood. Regulatory T cells (Treg) are pivotal for maintaining peripheral self-tolerance and controlling autoimmunity. This study was undertaken to examine the therapeutic effect of triptolide in lupus mice and the related molecular mechanisms. Our results showed that triptolide treatment ameliorated serum anti-dsDNA, proteinuria and renal histopathologic assessment in MRL/lpr mice, induced the miR-125a-5p expression and enhanced the proportion of Treg in vivo. In vitro, triptolide upregulated the proportion of Treg and the miR-125a-5p expression. Down-regulation of the miR-125a-5p expression reversed the effect of triptolide on Treg. In conclusion, triptolide could induce Treg and the miR-125a-5p expression in vivo and in vitro. Inhibiting the effect of miR-125a-5p could counteract the effect of triptolide on inducing Treg. The study has strong implications for the therapeutic applications of triptolide in systemic lupus erythematosus.

1. Introduction

Triptolide, a diterpene triepoxide extract from the Chinese herb Tripterygium wilfordii Hook F (TWHF), is a potent immunosuppressive compound. TWHF as a natural medicine can be dated back several centuries ago. It has been used for treatment of systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), nephritis, Behcet's disease, psoriasis, etc. [1]. In animal studies, triptolide significantly improved the renal function in lupus mice through suppressing the production of inflammatory mediators [2]. Triptolide inhibited the migration and invasion of rheumatoid fibroblast-like synoviocytes [3]. One of triptolide analogs (LLDT-8) had immunosuppressive activities in both cellular and humoral immune responses [4]. However, the specific mechanisms of triptolide in SLE have not been given sufficient attention.

SLE is a chronic autoimmune inflammatory disease characterized by a myriad of immunoregulatory abnormalities that lead to injuries of tissues and organs, which is caused by a failure of endogenous mechanisms of immune tolerance [5]. Regulatory T cells (Treg), expressing the transcription factor Foxp3, are pivotal for maintaining peripheral self-tolerance and controlling autoimmunity by suppressing the activation and expansion of autoreactive T cells and other pathogenic immune cells [6].

As microRNAs (miRNAs) are becoming increasingly recognized as negative regulators of gene expression [7], we suppose whether triptolide regulates Treg via miRNAs. Additionally, we have previously demonstrated that the expression of miR-125a-5p was significantly downregulated in SLE T cells , which is a critical factor that controls autoimmune diseases by stabilizing Treg-mediated immune homeostasis [8,9]. Here we show that triptolide upregulats miR-125a-5p and the proportion of Treg in spleen tissues of MRL/lpr mice, the most commonly studied mouse model for human lupus. Our study has uncovered a novel miRNA target of triptolide in treating SLE.

Triptolide was reported to inhibit lymphocyte activation and T cell expression of IL-2 at transcription level by inhibiting NF-κB transcriptional activation [10]. However, IL-2 deficiency results in a profound disturbance of Treg homeostasis and the development of a severe systemic autoimmune disease due to uncontrolled hyperactivity of T and B

* Corresponding author.
E-mail address: lingyunsun@nju.edu.cn (L. Sun).

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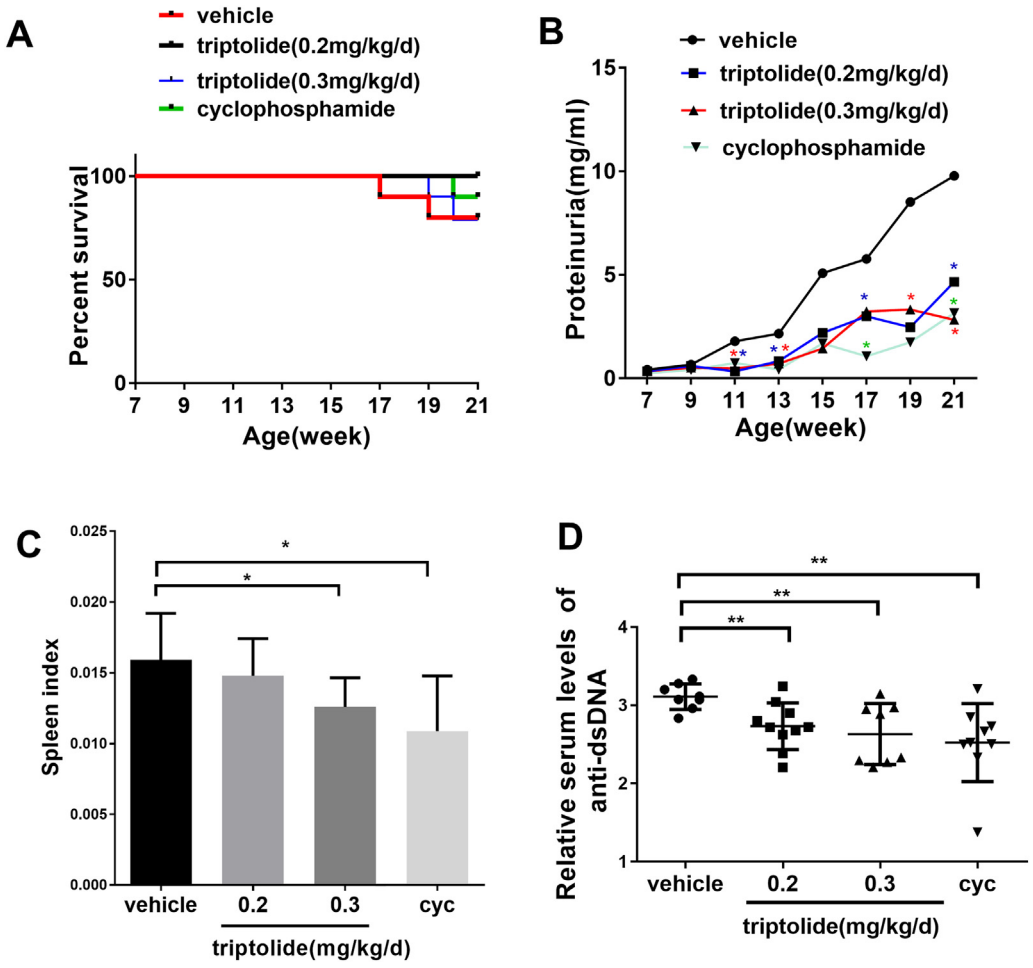

Fig. 1. Effects of treatment with triptolide on survival rate (A), proteinuria (B), spleen index (C) and serum anti-dsDNA level (D) in MRL/lpr mice. 7-week-old female MRL/lpr mice were orally administered vehicle (vehicle, $n = 8$), triptolide (0.2 mg/kg/d, n = 10), triptolide (0.3 mg/kg/d, n = 8) or cyclophosphamide (20 mg/kg/w, n = 9) daily by 21 weeks of age. A, survival rate in MRL/lpr mice from 7 to 21 weeks of age. B, the proteinuria levels were measured every two weeks. C, levels of spleen index (spleen weight/body weight) at the end of study. D, serum levels of IgG anti-dsDNA were detected by ELISA at the end of study. $^{*}P < 0.05$, $^{**}P < 0.01$, cyc = cyclophosphamide.

cells [11]. Therefore, the effects of triptolide on Treg have not been fully elucidated. In this study, we examined the role of triptolide in SLE and discussed the related mechanisms both in vivo and in vitro. We found that miR-125a-5p was down regulated in lupus, and triptolide could upregulate the expression of miR-125a-5p and the proportion of Treg. Importantly, triptolide elevated proportion of Treg via miR-125a-5p. This study provides novel insights into the mechanism underlying the therapeutic effect of triptolide on SLE.

2. Materials and methods

2.1. Animals

Female MRL/lpr mice and C57BL/6 (B6) mice were obtained from Shanghai Ling Chang Biological Technology Co., Ltd. (Shanghai, P.R. China). All mice were housed under specific pathogen-free conditions and kept in a 12 h light/dark cycle with controlled humidity (60%–80%) and temperature (22 ± 1 °C) in the animal center of the Affiliated Drum Tower Hospital of Nanjing University Medical School. All experiments were performed according to the institutional ethical guidelines on animal care and were approved by the Committee of Experimental Animal Administration of the Affiliated Drum Tower Hospital of Nanjing University Medical School.

2.2. Drug and administration protocol

For therapy, 40 female MRL/lpr mice at 7 weeks of age were randomly divided into 4 treatment groups. After one week adaptation to the environment, they were treated orally with vehicle (1% DMSO/1% Tween 20 in ddH_2O, $n = 10$), triptolide 0.2 mg/kg/d (n = 10), triptolide 0.3 mg/kg/d (n = 10) or cyclophosphamide (20 mg/kg/w, intraperitoneally, n = 10). Animals were sacrificed after 13 weeks of treatment. Mice were anaesthetized at the end of the experiments. Spleens were isolated and weighed, photographed and lymphocytes were prepared. Both kidneys were also excised for section analysis. Triptolide was purchased from Sigma (St. Louis, MO), which were ≥98% pure, as assessed by High Performance Liquid Chromatography (HPLC). Cyclophosphamide (Cyc) was purchased from Baxter Oncology GmbH. (license number: H20110407).

2.3. Histological analysis

The kidneys were fixed in 4% paraformaldehyde (PFA), dehydrated in graded alcohol, embedded in paraffin, sectioned at 3 μm, and stained with hematoxylin and eosin (H&E; Sinopharm Chemical Reagent, Shanghai, P.R. China). Histological scores of renal lesions were determined as described previously [12]. Briefly, the severity of glomerulonephritis was graded on a 1–4 scale as follows: 0, normal; 1, focal, mild, or early proliferative; 2, multifocal proliferative with increased matrix; 3, diffuse proliferative; 4, extensive sclerosis/crescents. Interstitial lesions were also graded on a 1–4 scale according to the number/foci of mononuclear cells around tubules. Perivascular cell accumulation was determined semiquantitatively by scoring the number of cell layers surrounding the majority of vessel walls on a 0–3 scale (0 = none; 1 ≤ 5 cell layers; 2 = 5–10 cell layers; 3 ≥ 10 cell layers). We evaluated renal pathology using coded slides.

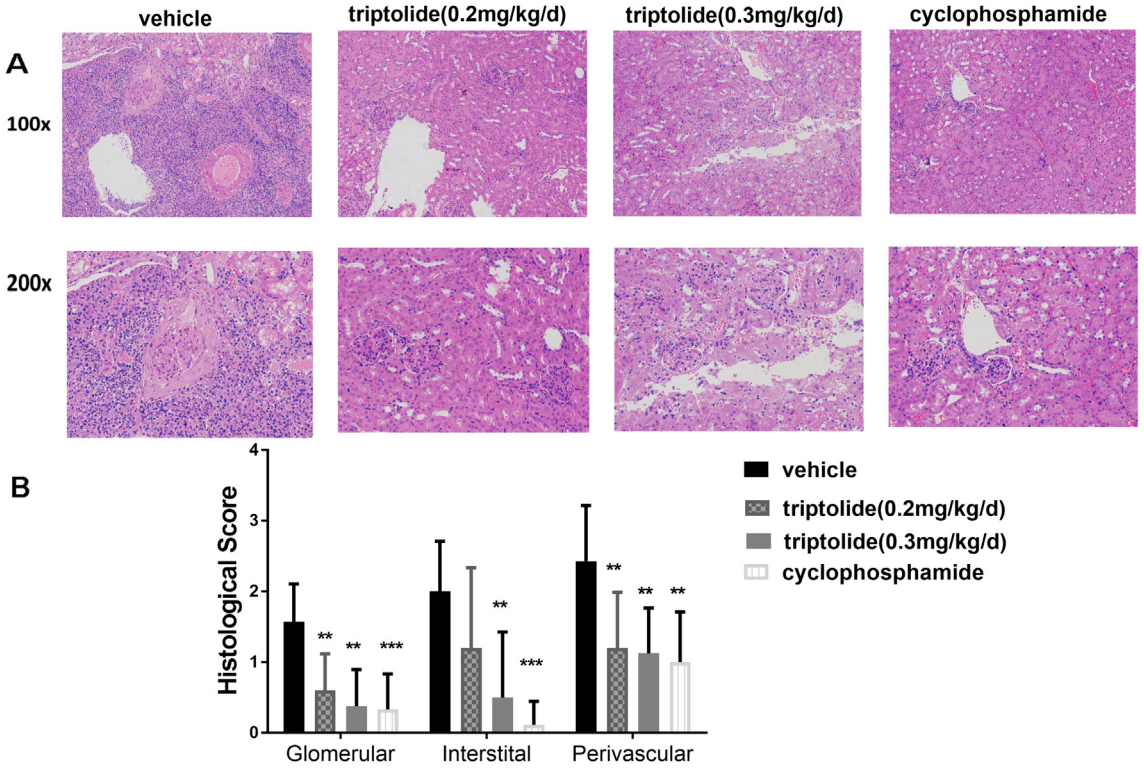

Fig. 2. Triptolide ameliorates lupus nephritis in MRL/lpr mice. A, Representative kidney sections stained with H&E (top panel, original magnification, ×100; bottom panel, original magnification ×200). B, Histological score for glomerulonephritis (Glome), interstitial nephritis (Intersti) and tubular casts, according to H&E staining. $^{**}P < 0.01$, $^{***}P < 0.001$ versus vehicle.

2.4. Anti-dsDNA antibody enzyme-linked immunosorbent assay (ELISA) and detection of the urinary protein

Anti-dsDNA Abs in the mice serum were determined by ELISA assay as previously described [13,14]. ELISA plates (Costar, Cambridge, MA) were pretreated with protamine sulfate (Sigma) and then coated with 50 mg/ml calf thymus dsDNA (Sigma). Post-incubation with mouse serum, the levels of anti-dsDNA Abs were detected with the HRP-conjugated goat anti-mouse IgG and IgM (Southern Biotechnology Associates, Birmingham, AL). Tetramethylbenzidine substrate was used to develop colors, and absorbance at 450 nm was measured on a microplate reader (Bio-Tek ELX800, Bio-Tek Instruments, Winooski, VT). The concentration of urine protein was determined wit Bradford Protein Assay Kit (KeyGen BioTECH, Nanjing, Jiangsu, China) according to the manufacturer's instructions.

2.5. Isolation and culture of mice splenocytes and lymph node cells

Freshly prepared spleens and perirenal lymph nodes were ground, and the splenic slurry was filtered through a 200-mesh sieve. Then the cell suspension was centrifuged at 400 ×*g* for 5 min and the cell pellet was resuspended with red cell lysis buffer and washed with PBS. Triptolide used in experiments in vitro was dissolved in 0.01% dimethyl sulfoxide (DMSO) and added to cells at the indicated concentrations. Briefly, splenocytes were treated with vehicle (0.01% DMSO), 1 nM triptolide or 10 nM triptolide in the presence of 1 μg/ml soluble anti-CD3 and anti-CD28 (eBioscience, San Diego, CA) in a 5% CO_2 humidified atmosphere and cultured in RPMI-1640 supplemented with 10% FBS (all from Gibco, Life Technologies) at 37 °C. After 72 h all the cells were analyzed as described below.

2.6. Flow cytometric analysis

Mice splenocytes were stained for surface markers with mouse FITC-anti-CD4, APC-anti-CD25 and then the cells were fixed and permeabilized with Cytofix/Cytoperm (eBioscience) and stained with PE-anti-Foxp3. All antibodies were purchased from Miltenyi Biotec or eBioscience. Flow cytometric analysis was performed on a FACSCalibur (BD Biosciences, Mountain View, CA) and data analysis was conducted using CellQuest software (BD Biosciences). Expression of Foxp3 was analyzed by gating on homogenous level of $CD4^+$ $CD25^+$cells.

2.7. Cell viability measurements

Splenocytes were plated on 96-well plates at 1.0×10^5/well and then treated with vehicle or triptolide at the indicated dosages for 72 h. The cell viability was evaluated by cell counting kit-8 (CCK-8) assay (KeyGEN, BioTECH, China).

2.8. RNA isolation and quantitative reverse transcription PCR

To quantify miR-125a-5p, total RNA was obtained from cells using TRIzol (Takara, Dalian, Liaoning, P.R. China) and cDNA was synthesized using reverse transcription by Mir-X™ miRNA First-Strand Synthesis Kit (Takara). For real time PCR experiments, reactions containing the SYBR Premix EX TaqTM (Takara), ROX Reference Dye (50×, Takara), cDNA and gene primers were run on the StepOnePlus Real Time PCR Systems (Applied Biosystems, USA) and analyzed with StepOne Soſtwase V2.1 (Applied Biosystems). The reverse transcription product was amplified using primer pairs specific for miR-125a-5p. U6 was used as controls for quantification. To quantify the mRNA, collected cells in TRIzol (Takara, Dalian, Liaoning, P.R. China), and cDNA was synthesized using reverse transcription by PrimeScript RT Master Mix (Takara). Transcripts of human Foxp3 were detected via AceQqPCR SYBR Green Master Mix (Vazyme) according to the manufacturer's instructions. Primer sequences were as follows: GAPDH:5′-ACAACTTTG GCATTGTGGAA-3′ (forward), 5′-GATGCAGGGATGATGTTCTG-3′ (reverse); Foxp3: 5′-CACCTATGCCACCCTTATCCG-3′ (forward), 5′-CATG

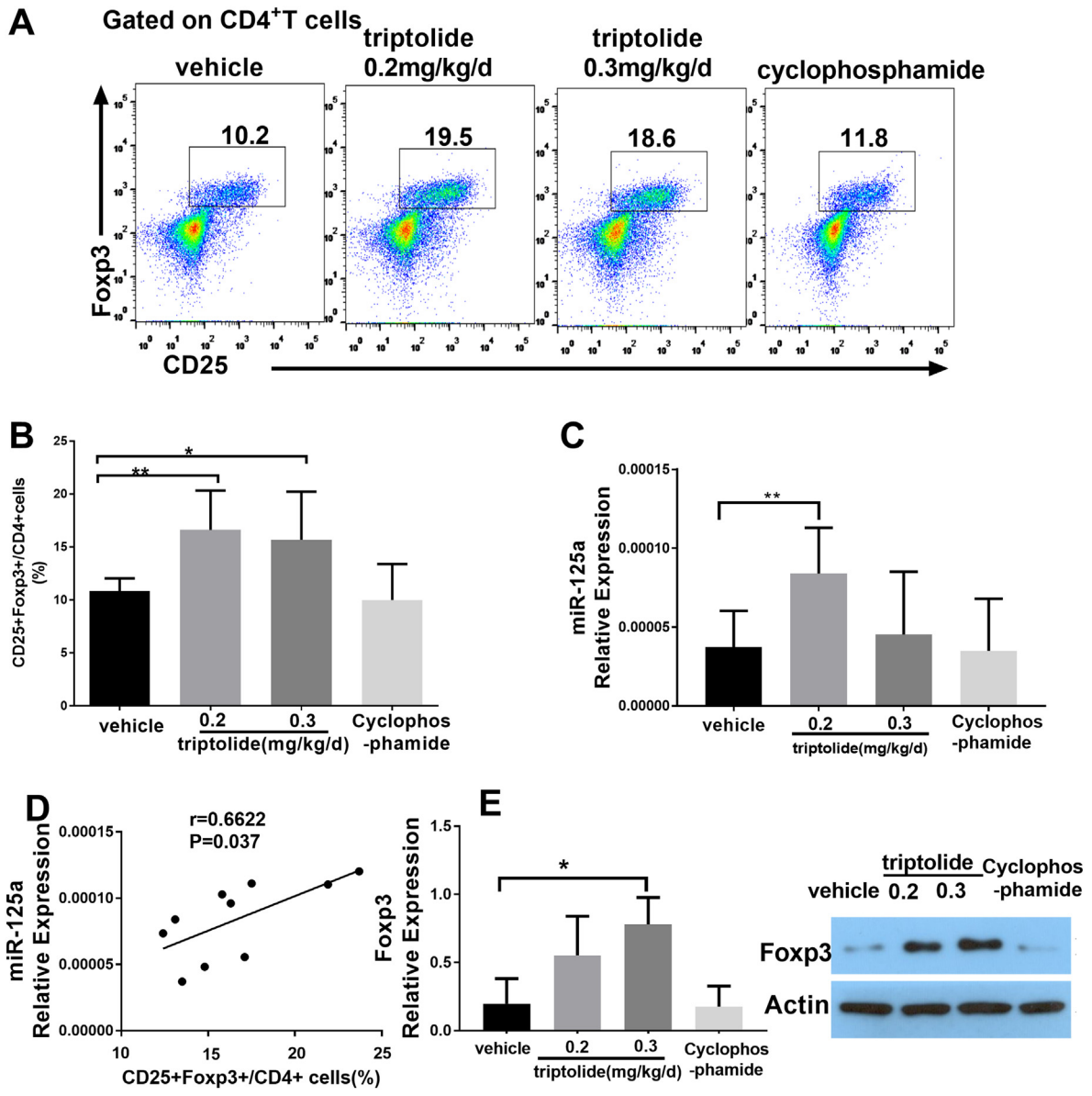

Fig. 3. Changes in Treg proportion and miR-125a-5p expression after treatment with triptolide in MRL/lpr mice. A, Representative staining of Treg ($CD4^+CD25^+Foxp3^+$) cells among $CD4^+$ T splenocytes. B, Statistical results of percentage of Treg ($CD4^+CD25^+Foxp3^+$) population in splenocytes of 4 groups. C, The expression of miR-125a-5p in splenocytes of MRL/lpr mice D, The correlation between the proportions of Treg and miR-125a-5p expression in triptolide (0.2 mg/kg/d)-treated mice (n = 10 for each group) E, The expression of Foxp3 protein in spleen of MRL/lpr mice, as determined by Western blotting, Anti-actin was used as an internal control. Results are from 3 representative MRL/lpr mice. $^*P < 0.05$; $^{**}P < 0.01$.

CGAGTAAACCAATGGTAGA-3′ (reverse). Threshold cycle (CT) values were measured and the relative abundance of the miRNA was calculated with the $2^{-\Delta\Delta Ct}$ method.

2.9. Transfection of miR-125a-5p inhibitor

MicroRNA oligonucleotides were obtained from GenePharma (Shanghai, China). MiRNA hairpin inhibitors are single-stranded oligonucleotides for downregulation of miRNA expression. Mice splenocytes were seeded in 24-well plates (1.5×10^4 cells/well) and incubated overnight prior to transfection. Transfection mastermixes were diluted in Opti-MEM (Life Technologies, Carlsbad, CA) containing HiPerFect (Qiagen, Valencia, CA), miR-125a-5p inhibitor or negative control (NC). Transfected cells were analyzed for fluorescence activated cell sorting (FACS).

2.10. Western blotting

Total protein samples of spleen were isolated by 1× radioimmunoprecipitation assay (RIPA) lysis buffer with 1% protease inhibition (Beyotime, China). The concentration of protein samples was measured using a BCA method (Beyotime, China). Protein samples were separated by 12% sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride membranes (Millipore). After blocking with 5% non-fat milk in TBST (10 mM Tris (BioSharp, Hefei, Anhui, China) (pH 7) and 150 mM NaCl, 0.1% Tween 20), and immunoblotted with Foxp3 (Servicebio, China), and Actin (Servicebio, China) primary antibodies, and immunoblotted with the goat anti-rabbit secondary antibodies. The protein bands were visualized using an enhanced chemiluminescence reagent (Beyotime, China) and the bands were visualized by a G:BOX gel imaging system (Syngene, Cambridge, UK). Analysis was performed by using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

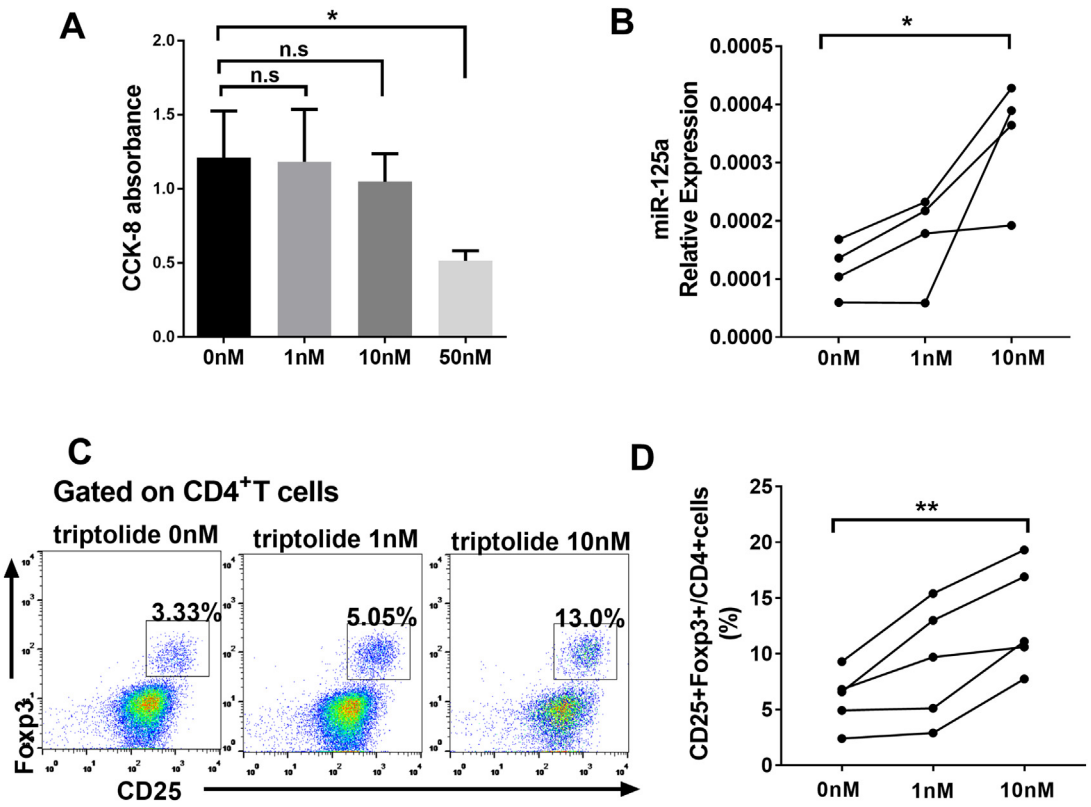

Fig. 4. Triptolide induced mouse Treg proportion and miR-125a-5p expression in vitro. A, The viability of splenocytes was evaluated when cultured with different concentrations of triptolide (0 nM–50 nM) by cell counting kit-8 (CCK-8) assay B, Triptolide induced miR-125a-5p production in splenocytes, as determined by RT-PCR. C, Representative staining of Treg ($CD4^+CD25^+Foxp3^+$) cells among $CD4^+$ T splenocytes after cocultured with triptolide. D, Statistical results of percentage of Treg ($CD4^+CD25^+Foxp3^+$) in splenocytes of 0 nM, 1 nM and 10 nM triptolide, respectively ($n = 5$). Data are representative of three to five separate experiments with similar results. $^*P < 0.05$, $^{**}P < 0.01$, n.s = no significant difference.

2.11. Statistical analysis

The data were expressed as mean ± standard error of mean (SEM) and analyzed with Prism 5 (GraphPad Software). A two-tailed Student's *t*-test was applied for statistical comparison of two groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Triptolide ameliorated the clinical symptoms of MRL/lpr mice

Female MRL/lpr mice develop spontaneously that closely resemble human SLE as early as 8 weeks of age and is characterized by the production of various autoantibodies and the development of fatal glomerulonephritis [15]. The preventative effects of triptolide were tested in MRL/lpr mice from 8 to 21 weeks of age. Vehicle mice group began dying at 17 weeks of age, and by 21 weeks, the cumulative mortality was 20% (2 of 10). In contrast, there was no death in triptolide (0.2 mg/kg)-treated mice at the end of the study (Fig. 1A). As shown in Fig. 1B, 7-week-old MRL/lpr mice (before treatment) were free of overt disease, with only low levels of proteinuria. The mean level of urinary protein was not significantly different at 7 weeks of age in the 4 groups (Fig. 1B). With aging, proteinuria in vehicle-treated mice increased progressively. However, the onset of severe proteinuria was significantly delayed in both triptolide-treated and cyclophosphamide-treated mice. Proteinuria was significantly lower in triptolide-treated mice compared with vehicle-treated mice at 21 weeks of age (Fig. 1B).

Splenomegaly and lymphoproliferation are considered to be clinical markers of lupus patients and MRL/lpr mice. By the end of the study, mice were sacrificed. Spleen index (ratio of spleen weight to body weight) was reduced significantly after treatment with triptolide (0.3 mg/kg/d) and cyclophosphamide compared with vehicle (Fig. 1C). Weight of perirenal lymph nodes were detected is not significant among four groups as shown in Supplementary Fig. 1A. In addition, results showed that triptolide (0.2 mg/kg/d and 0.3 mg/kg/d) and cyclophosphamide treatment significantly decreased serum anti-dsDNA levels (Fig. 1D).

3.2. Triptolide ameliorated the histological symptoms of MRL/lpr mice

Kidney histopathology confirmed that the mice in the vehicle group exhibited severe renal injury, which was characterized by glomerulosclerosis, crescent formation, tubular cast deposition, increased mesangial matrix, and diffuse perivascular and interstitial mononuclear cell infiltration. As shown in Fig. 2A and B, renal damage was alleviated in mice treated with triptolide and cyclophosphamide, and in particular there was a significant reduction in glomerular hypercellularity, glomerulosclerosis, renal interstitial and perivascular lesions in triptolide (0.3 mg/kg/d) and cyclophosphamide groups. Pathological features were also ameliorated significantly in glomerular hypercellularity, glomerulosclerosis and perivascular lesions in triptolide (0.2 mg/kg/d)-treated mice.

3.3. The proportion of Treg and the level of miR-125a-5p increased in triptolide-treated MRL/lpr mice

As an important suppressor cell of immune system, Treg are important in modulating the immune homeostasis as well as tolerance to self-antigens. In the present study, splenocytes were obtained from 21-week-old female MRL/lpr mice after treatment. As shown in Fig. 3A and B, the proportion of $CD4^+CD25^+Foxp3^+$Treg subset increased significantly in the triptolide-treated mice compared with vehicle-treated

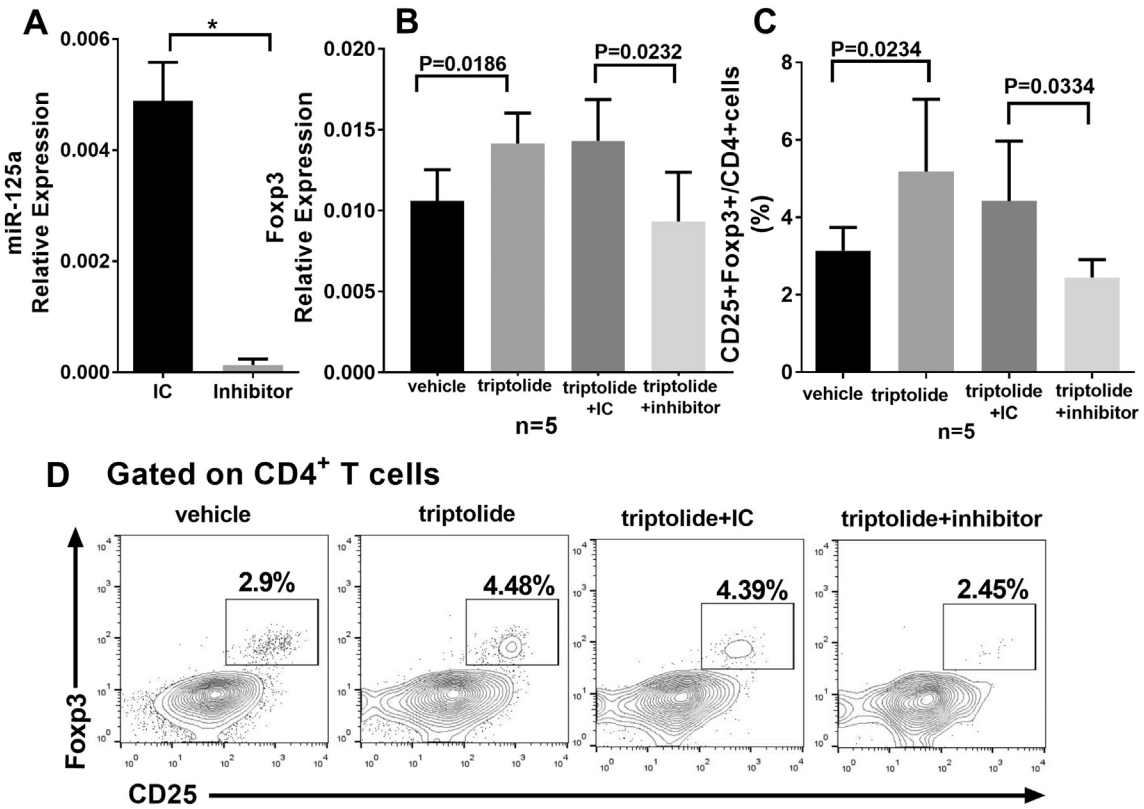

Fig. 5. Identification of miR-125a-5p as a regulator of Treg after triptolide treatment. A, MiR-125a-5p expression in splenocytes transfected with either miR-125a inhibitor or inhibitor control (IC), as determined by RT-PCR. B, Foxp3 mRNA in splenocytes treated with triptolide or vehicle were detected by RT-PCR. Statistical results of Foxp3 in splenocytes treated with 10 nM triptolide and transfected with either miR-125a-5p inhibitor or inhibitor control. C, Treg proportions in splenocytes treated with triptolide or vehicle were detected by FACS. Statistical results of percentage of Treg proportions in splenocytes treated with 10 nM triptolide and transfected with either miR-125a-5p inhibitor or inhibitor control. D, Contour Plot showed Treg proportion in splenocytes treated with triptolide or vehicle and transfected with either miR-125a-5p inhibitor or control mimic. Data are representative of five separate samples with similar results. *$P < 0.05$. IC = inhibitor control; inhibitor = miR-125a-5p inhibitor.

mice. As shown in Supplementary Fig. 1B, statistical results of percentage of Treg population in lymphocyte of 4 groups of lymph nodes are similar to that in spleens.

Previous study demonstrated miR-125a-5p as a critical regulator that stabilizes Treg-mediated immune homeostasis and controls autoimmunity. To evaluate whether the expression of miR-125a-5p was influenced in triptolide-treated group, we assayed miR-125a-5p expression via real-time PCR in splenocytes. The results showed that miR-125a-5p was significantly upregulated in triptolide (0.2 mg/kg/d)-treated group (Fig. 3C). Especially, the expression of miR-125a-5p is positively correlated with the proportions of Treg in triptolide (0.2 mg/kg/d)-treated mice (Fig. 3D).

As Foxp3 is not only an important molecular marker of Treg but also a key transcription factor that affects Treg function and development, we detected Foxp3 protein levels in spleens of four groups of MRL/lpr. The results showed Foxp3 protein was significantly upregulated in triptolide (0.3 mg/kg/d)-treated group. The Foxp3 protein was upregulated in triptolide (0.2 mg/kg/d)-treated group, but not significantly (Fig. 3E).

3.4. Triptolide increased Treg percentage and miR-125a-5p expression in vitro

As shown in Fig. 4A, dose-dependent CCK-8 experiments (from 0 nM to 50 nM) revealed that 1 nM and 10 nM triptolide did not influence the proliferation of splenocytes until 50 nM triptolide. So in the following experiments 1 nM and 10 nM triptolide were used. To confirm the effect of triptolide on miR-125a-5p expression and Treg in vitro, splenocytes isolated from B6 mice were treated with vehicle or triptolide (0 nM or 10 nM) and cultured in the presence of anti-CD3 and anti-CD28. After cocultured for 3 days, splenocytes treated with triptolide showed an increase of miR-125a-5p expression in a dose-dependent manner compared with vehicle group. As shown in Fig. 4B, in the presence of 10 nM triptolide, miR-125a-5p levels in splenocytes increased significantly ($P < 0.05$). The frequency of $CD4^+CD25^+Foxp3^+$Treg was significantly upregulated in a dose-dependent manner (Fig. 4C and D).

3.5. Down-regulation of miR-125a-5p reversed the effect of triptolide on Treg

To verify the role of miR-125a-5p in the induction of Treg expression by triptolide, miR-125a-5p inhibitor was transfected into the splenocytes. The level of miR-125a-5p after transfection with miR-125a-5p inhibitor or inhibitor control (IC) was determined to verify the transfection effect. Results showed that the expression of miR-125a-5p markedly reduced after transfecting with miR-125a-5p inhibitor (Fig. 5A). Compared with the vehicle group, 10 nM triptolide enhanced the Foxp3 mRNA expression, as shown in Fig. 5B. When miR-125a-5p inhibitor was transfected into the triptolide-treated splenocytes, the Foxp3 mRNA reduced significantly compared with inhibitor control. Compared with the vehicle group, 10 nM triptolide enhanced the proportion of Treg, as shown in Fig. 5C and D. When miR-125a-5p inhibitor was transfected into the triptolide-treated splenocytes, the proportion of Treg reduced significantly compared with inhibitor control. It means that miR-125a-5p inhibitor can reverse the effect of triptolide on Treg. These data suggested that the inhibition of miR-125a-5p in splenocytes could significantly abrogate the induction of Treg by triptolide. Taken together, triptolide induced Treg via miR-

125a-5p.

4. Discussion

Triptolide was first isolated from the medicinal plant TWHF and structurally elucidated in 1972 [16]. It has been widely used as one of the most common immunosuppressive agents for SLE in China mainly due to its favourable cost-benefit ratio, but the studies of triptolide treatment of lupus and related molecular mechanism in vivo are few. In this study, we explored the therapeutic effect of triptolide on a murine model of lupus and its new therapeutic mechanism. Results of the studies have shown that TWHF ameliorates immune nephritis in MRL/lpr mice [2,17]. One recent study found that triptolide ameliorated skin damage, suppressed the serum levels of IFN-γ and IL-10 in MRL/lpr mice and downregulated the mRNA level of TLR9, TLR4 and NF-κB in MRL/lpr mice [18]. Our study showed that triptolide-treated MRL/lpr mice have significantly decreased rates of mortality, dsDNA, lupus nephritis evidenced by proteinuria and renal histopathologic assessment, compared with vehicle-treated mice. It added further evidence to support a clinical trial of triptolide in the therapeutic efficacy of SLE. Overall, these data suggest that triptolide treatment can inhibit the activity and progress of SLE and thereby prolong survival.

MiRNAs have emerged as important regulators in many physiological and pathological processes [19]. The role of miRNA in the immune system has been highlighted to maintain immune system homeostasis [20]. Accordingly, dysregulated levels of miRNA are associated with a growing list of diseases, including autoimmune diseases [21,22]. Treg cells expressing the transcription factor Foxp3 have emerged as dedicated suppressors of diverse immune responses and inflammation and vitally important keepers of immune homeostasis. Foxp3 binds to multiple genes and acts as a lineage specification factor of Treg cells [23]. Sustained expression of Foxp3 amplifies and stabilizes molecular features of Treg precursor cells and represses the molecular features deleterious to Treg cell differentiation or function. Thereby Foxp3 is required for Treg's differentiation and suppressor function [24,25]. Studies from ours and others have shown that miR-125a-5p is downregulated in T cells of SLE patients and lupus-prone MRL/lpr mice and it identified miR-125a-5p as a regulator that stabilizes the commitment and immunoregulatory capacity of Treg, consequently for the suppression of inflammation [8,9]. The numeric and functional deficits of Treg have been described in SLE patients and in lupus mice [11,26,27]. Treg numbers decrease in lupus-prone mice as the age and the disease progresses [28]. In vivo studies, we found that enhanced Treg proportion and miR-125a-5p expression after triptolide treatment, suggesting the possibility of beneficial effects of miR-125a-5p induction in the increase of Treg function after triptolide treatment in lupus. Further investigations showed a positive correlation between miR-125a-5p expression and Treg frequency. This suggests that the mechanism of triptolide therapy for lupus may be related to the increased expression of miR-125a-5p and Treg proportion. Our results are consistent with previous study that T2, whose active ingredient is triptolide, enhances in situ level of $Foxp3^+$ regulatory cells in Crohn's disease [29].

The induction expression of miR-125a-5p may partly result in expansion of Treg cells after treatment of triptolide in MRL/lpr mice, which was verified by our in vitro experiments. Of note, we found that after inhibition of miR-125a-5p expression in triptolide-treated cells, the proportion of Treg was declined significantly compared with control group.

Triptolide was reported to inhibit lymphocyte activation and T cell proliferation of IL-2 at the level of transcription by inhibiting NF-κB transcriptional activation [30]. However, impaired IL-2 production by T cells has also been attributed a critical role in murine and human lupus. IL-2 deficiency results in a profound disturbance of Treg homeostasis and the development of a severe systemic autoimmune disease due to uncontrolled hyperactivity of T and B cells [31,32]. It is seems a contradiction in the treatment of SLE. For the first time we found that triptolide induced miR-125a-5p expression, further enhanced the proportion of Treg and treated murine lupus. Studies show that miR-125a-5p directly suppressed several targets, including Stat3, which are critical factors of effector lineages and also detrimental to Treg differentiation [8]. This is in agreement with previous study that triptolide suppressed the Stat3 signaling pathway [33]. In conclusion, we propose a new model on how triptolide induce the proportion of Treg via miR-125a-5p in lupus-prone MRL/lpr mice.

Despite triptolide remarkable effect on lupus mice, an increasing number of studies demonstrated that it could induce toxicity [34]. The clinical applications of triptolide are restricted by its side effects. In our study, 1 nM and 10 nM triptolide did not influence the viability of splenocytes in vitro. There is no death in mice treated with triptolide (0.2 mg/kg/d) in vivo. Therefore, further studies regarding the toxicity of triptolide concentration in clinical settings are necessary.

In conclusion, triptolide alleviated SLE through miR-125a-5p mediated upregulation of Treg proportion. We propose a new model on how triptolide induced Treg in lupus-prone MRL/lpr mice, which may prove to have strong implications in guiding the therapeutic applications of triptolide in the treatment of SLE and other autoimmune diseases.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.02.047.

Disclosure of interest

The authors declare that there is no conflict of interests.

Acknowledgments

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