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Research Article
Therapeutic Effects of Triptolide on Lupus-prone MRL/lpr Mice

[1]Xueqin Huang, [2]Chengping Wen and [1]Hua Wei

[1]Zhejiang Key Laboratory of Pathophysiology, Department of Immunology, Medical School of Ningbo University, Ningbo, 315211 Zhejiang, China
[2]College of Basic Medical Science, Zhejiang Chinese Medical University, Hangzhou, 310053 Zhejiang, China

Abstract

Background and Objective: Systemic lupus erythematosus (SLE) is a multisystem autoimmune disease characterized by various immunological abnormalities. Triptolide is a diterpene triepoxide antibiotic compound that can be isolated from extracts of the medicinal plant, *Tripterygium wilfordii* Hook F., which has been used for a number of years in traditional Chinese medicine. Triptolide have immunosuppressive and anti-inflammatory properties. The present study aimed to determine the effects of triptolide on lupus-prone MRL/lpr mice. **Materials and Methods:** MRL/lpr mice were divided into two groups, including model control group and triptolide-treated group. Mice were administered for 8 weeks by oral gavage. Eight weeks after treatment, the mice serum levels of interferon-γ (IFN-γ) and interleukin-10 (IL-10) were measured with ELISA. The kidney damage of MRL/lpr mice was examined with haematoxylin and eosin (HE) staining and immunofluorescence of deposition of IgG and IgM in glomeruli. Gene expression levels of Toll-like receptor (TLR9), Toll-like receptor 4 (TLR4) and Nuclear factor-κB (NF-κB) in kidney of MRL/lpr mice were measured using real-time PCR. One-way ANOVA, followed by Newman-Keuls test or Student's t-test were used. **Results:** The results showed that triptolide improved skin damage, decreased the serum levels of IFN-γ and IL-10 in MRL/lpr mice. Moreover, triptolide could improve renal histopathologic characteristics of MRL/lpr mice and downregulate the mRNA level of TLR9, TLR4 and NF-κB. **Conclusion:** These findings indicated that triptolide might have the potential therapeutic utility for the treatment of SLE.

Key words: Triptolide, lupus, MRL/lpr mice, skin damage, kidney damage

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Corresponding Author: Xueqin Huang, Zhejiang Key Laboratory of Pathophysiology, Department of Immunology, Medical School of Ningbo University, Ningbo, 315211 Zhejiang, China Tel: 86-574-87600763

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a prototype autoimmune disease characterized by aberrant immune regulation and excessive production of auto antibodies leading to intense inflammation and multiple organ damage[1-3]. Deposition of circulating auto antibody-autoantigen complexes can occur in various tissues and organs of the body, resulting in a local inflammatory response and severe tissue destruction. Sites often affected include skin, the kidney, the nervous system, joints and muscles, which contribute most significantly to disease morbidity[4,5].

MRL/lpr mice as one of the commonly used animal models of SLE, symptoms and human lupus are similar, including significantly higher levels of serum antibodies and immune complex in glomerular nephritis and so on[6,7]. To discuss the pathogenesis, pathology and treatment method have important meaning. Because of the resemblance between the murine and human diseases, MRL/lpr mice have been used extensively to determine SLE etiology and to evaluate therapies. Indeed, MRL/ lpr mice provide an attractive model because their syndrome is spontaneous, predictable and rapid; it exhibits the characteristic multifaceted tissue destruction and sexual dimorphism[8-10].

For many years, SLE standard therapy included antimalarials, steroids and immunosuppressive drugs. Though efficient in improving quality of life, survival and well-being and maintaining long remissions, they are still associated with many side effects, some of which are severe[4,11,12]. Therefore, new, novel and better focused therapies are needed. Triptolide, which is a diterpenoid, is the main active component of the *Tripterygium wilfordii* plant, which is a traditional Chinese herb and used for a number of years in traditional Chinese medicine[13,14]. Previous studies have shown that triptolide has multiple pharmacological activities, including anti-inflammatory, immune modulation, antiproliferative and proapoptotic activity[15-18]. In the current study, it was aimed to investigate the therapeutic effects of triptolide in MRL/lpr mice, including improving skin lesions, renal pathology and serum cytokines.

MATERIALS AND METHODS

This study was carried out between 2014 and 2015 in the Laboratory of Animal Experiments and Research Laboratory, College of Basic Medical Science, Zhejiang Chinese Medical University, Hangzhou, Zhejiang, China.

Materials and reagents: Triptolide were obtained from Institute for drug control (Hangzhou, Zhejiang province, China). The antibodies of FITC-conjugated donkey anti-mouse IgG and FITC-conjugated goat anti-mouse IgM were obtained from sangon (Shanghai, China). IFN-γ and IL-10 ELISA kit were purchased from eBioscience (San Diego, CA, USA).

Mice and treatments: Female MRL/lpr mice aged 8 weeks were purchased from Experimental Animal Center of Shanghai, Chinese Academy of Sciences, bred and maintained in the Laboratory of Animal Experiments at Zhejiang Chinese Medical University. All mice were accustomed in SPF standards animal laboratory for 1 week before experiments started. Sixteen female MRL/lpr mice were divided randomly into two groups: Model control group and triptolide-treated group. Each group contained 8 mice. Mice of the triptolide-treated group were administered with triptolide 100 μg kg^{-1}/day. Mice of the model control group were treated with normal saline by oral administration. Treatment was administered by oral gavage up to 16 weeks of age.

Histopathology and immunohistochemistry: Kidney samples were fixed overnight in 4% paraformaldehyde, then dehydrated and embedded in paraffin. Thin sections (3 mm) were stained with hematoxylin and eosin (HE) by standard methods and the kidney impairment was examined by a light microscopy.

For immunohistochemical labeling, kidney samples were frozen in OCT compound, frozen kidneys were cut into 5 mm sections, fixed in acetone, rinsed with PBS and incubated with the antibodies of FITC-conjugated donkey anti-mouse IgG diluted 1:100 or FITC-conjugated goat anti-mouse IgM diluted 1:100, rinsed with PBS. The photomicrographs were obtained using a fluorescence microscopy (BX43, Olympus).

ELISA detection of IFN-γ and IL-10: To determine the release of inflammatory cytokines, IFN-γ and IL-10 were measured using a Platinum ELISA kit (eBioscience) according to the manufacturer's instructions.

Real-time PCR: RNA samples were reverse transcribed into cDNA using SuperScript II reverse transcriptase (Takara Bio, Shiga, Japan) and oligo (dT) primers. To determine the quantity of mRNA, the cDNA was amplified by real-time PCR with a SYBR Premix Ex *Taq* RT-PCR kit (Bio-Rad, Hercules, CA) and GAPDH was used as the internal control. Relative expression levels were calculated using the $2^{-\Delta\Delta}$Ct method. The following primers were used: Mouse TLR9 (forward:

5′-TTCTCAAGACGGTGGATC-3′, reverse: 5′-GGCGCAGTCGCAC ATAG-3′), mouse TLR4 (forward: 5′-CATTCAAGACCAAGCC TTTCAG-3′, reverse: 5′-CCAGGTTTTGAAGGCAAGTTTT-3′), mouse NF-κB (forward:5′-AGGCTTCTGGGCCTTATGTG-3′, reverse: 5′-TGCTTCTCTCGCCAGGAATAC-3′) and mouse GAPDH (forward: 5′-TGC ACC ACC AAC TGC TTA G-3′, reverse: 5′-GGA TGC AGG GAT GAT GTT C-3′).

Statistics analysis: Data were expressed as Mean±SEM from at least three independent experiments. Differences between groups were evaluated with one-way ANOVA, followed by Newman-Keuls test or Student's t-test. Statistical analysis was performed using Prism 5.0 (GraphPad Software, San Diego, CA, USA).A significance level of $p<0.05$ was considered statistically significant.

RESULTS

Blocking of SLE development by triptolide in MRL/lpr mice: To evaluate whether treatment with triptolide have an effect on MRL/lpr mice, firstly skin destruction was analyzed in both model control group and triptolide-treated group. Facial and body skin in MRL/lpr model control group mice administered normal saline were obviously damaged. In contrast, mice treated with triptolide developed significantly less damage to facial and body skin.

Triptolide decreased the serum level of IFN-γ and IL-10: To assess whether triptolide improves typical clinical symptoms of SLE, it was evaluated the levels of IFN-γ and IL-10. It was compared concentrations in peripheral blood serum of MRL/lpr mice treated with triptolide and normal saline, IFN-γ and IL-10 was significantly reduced in 100 μg kg^{-1}/day triptolide-treated mice compared with control group mice administered normal saline (Fig. 1).

Ameliorated glomerulonephritis by triptolide in MRL/lpr mice: The kidneys of the mice at 16 weeks showed typical glomerulonephritis, characterized by enlarged glomeruli, proliferation of glomerular cells, the expansion of the mesangial extracellular matrix (ECM), infiltrating lymphocytes, neutrophils cells. In contrast, mice treated with triptolide showed a lesser degree of glomerulonephritis, varying degree of focal glomerular cell proliferation, only a slight increase in the ECM, reduction in the number of inflammatory cells (Fig. 2).

Deposition of IgG and IgM in glomeruli is a characteristic feature of the glomerulonephritis observed in MRL/lpr mice. However, in contrast to the intense deposition of IgG and IgM in the glomeruli of control group MRL/lpr mice, histologic analysis of the glomeruli of triptolide group MRL/lpr mice revealed only mild to moderate deposition of IgG and no or very little IgM deposition (Fig. 3, 4).

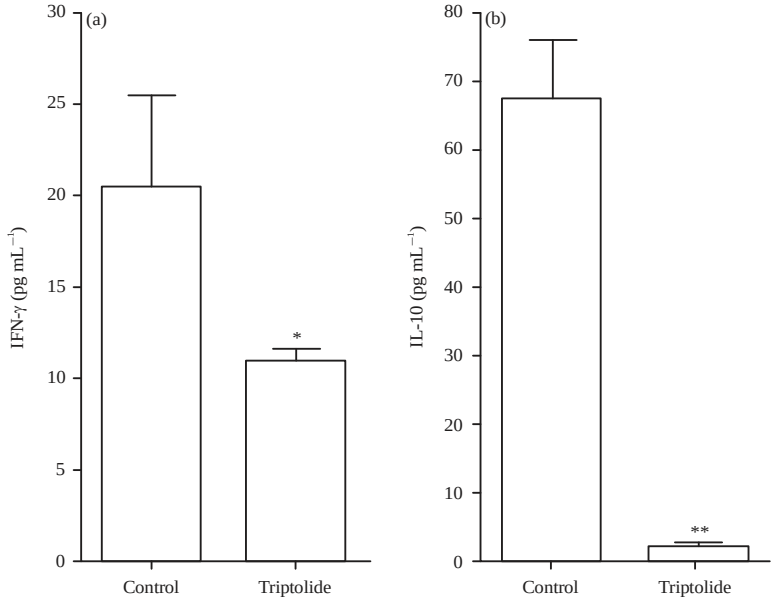

Fig. 1(a-b): Levels of IFN-γ and IL-10 in the two groups of MRL/lpr mice. The (a) IFN-γ and (b) IL-10 were determined in the MRL/lpr mice at 16 weeks of age

Values are expressed as Mean±SEM from at least three independent experiments using peripheral blood serum from one mouse per separate experiment *$p<0.05$ and **$p<0.01$ compared with control group

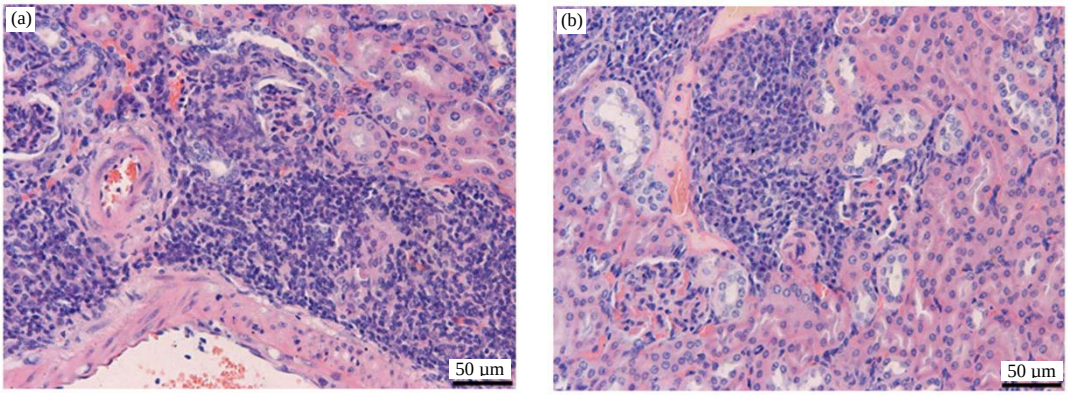

Fig. 2(a-b): HE staining of kidney sections in the two groups of MRL/lpr mice. (a) Control group mice at week 16 showed typical signs of severe glomerulonephritis and (b) Mice treated with triptolide showed a lesser degree of glomerulonephritis
The scale bar refers to 50 μm

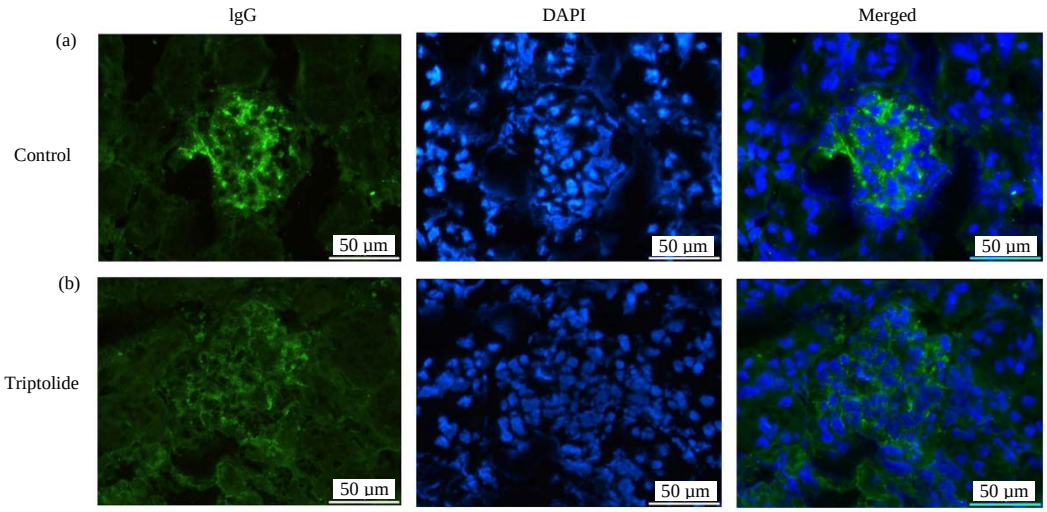

Fig. 3(a-b): Deposition of IgG in kidney as measured by direct immunofluorescence in (a) Control and (b) Triptolide-treated 16-weeks-old MRL/lpr female mice. Images are representative of eight mice in each group. Note that milder extent of deposition of IgG in the mesangium and capillary loop of glomeruli in triptolide-treated MRL/lpr mice
The scale bar refers to 50 μm

Triptolide downregulates the mRNA level of TLR9, TLR4 and NF-κB in kidney of MRL/lpr mice: To investigate whether triptolide alters the gene expression of TLR9, TLR4 and NF-κB in kidney of MRL/lpr mice, it was measured the mRNA levels of TLR9, TLR4 and NF-κB using real-time PCR. The results show the mRNA levels of TLR9, TLR4 and NF-κB in kidney of MRL/lpr mice were significantly down-regulated in 100 μg kg^{-1}/day triptolide-treated mice compared with control group mice administered normal saline (Fig. 5).

DISCUSSION

In the present study, the results showed that triptolide could improve the symptom of MRL/lpr mice. It maybe has a therapeutic effect on SLE. SLE is a heterogeneous chronic inflammatory autoimmune disorder, which is characterized by progressive involvement of multiple-organ systems with alternating clinical exacerbations and remissions[1,2]. The corticosteroids exert broad inhibitory effects on immune

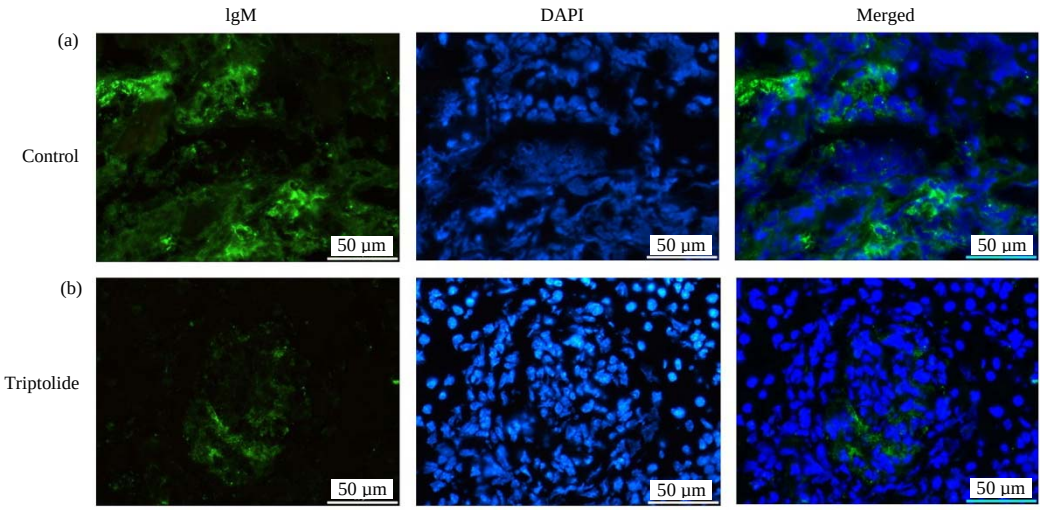

Fig. 4(a-b): Deposition of IgM in kidney as measured by direct immunofluorescence in (a) Control and (b) Triptolide-treated 16-weeks-old MRL/lpr female mice. Images are representative of eight mice in each group. Note that less extent of deposition of IgM in the mesangium and capillary loop of glomeruli in triptolide-treated MRL/lpr mice
The scale bar refers to 50 µm

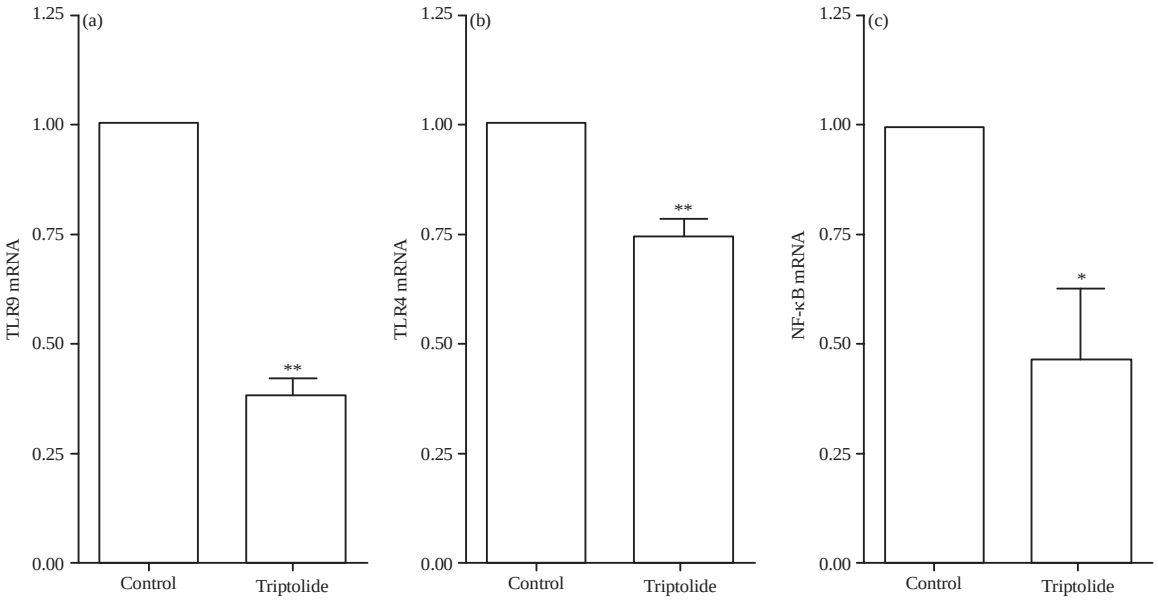

Fig. 5(a-c): Triptolide down regulates (a) TLR9, (b) TLR4 and (c) NF-κB gene expression in kidney of MRL/lpr mice. The mRNA was isolated from kidney of MRL/lpr mice and TLR9, TLR4 and NF-κB gene expression levels were determined using real-time PCR
Results are presented as the Mean ± SEM from at least three independent experiments using kidney from one mouse per separate experiment, **p<0.01 vs. Control and *p<0.05 vs. control

responses mediated by T and B cells and their rapid onset of actions made them widely used in managing acute SLE manifestations. However, the adverse effects involve infections, myalgias, osteoporosis, bone necrosis and cardiovascular disease[4,11]. The symptoms of MRL/lpr mice are similar to SLE. Therefore, it is important significance to investigate the therapeutic utility of triptolide on lupus-prone MRL/lpr mice.

In this study, it was demonstrated that triptolide significantly attenuated the disease phenotype of MRL/lpr mice after 8 weeks of drug therapy. Firstly, skin damage was analyzed in both model control group and triptolide-treated group and the result showed that mice treated with triptolide developed significantly less damage to facial and body skin than control group mice administered normal saline. Furthermore, there were significant differences in the IFN-γ and IL-10 between the mice treated with triptolide and untreated mice (Fig. 1). Some studies have shown that IFN-γ played the key role in the mechanism of SLE[19-21]. IFN-γ is a kind of typical Th1 type cytotoxin, it can aggravate SLE by improve cellular immunity and enhance the B cell activation. IFN-γ-producingTh1, IL-4-producing Th2 and IL-17A-producing Th17 cells[22-24], are the three major types of Th cells studied extensively. The results of this study compared with the model control group, the IFN-γ level of triptolide-treated group was significantly lower than that of model control group (Fig. 1a). Thus, IFN-γ also plays an important role in the worsening of the SLE. The result is consistent with previous findings, therefore, triptolide can adjust the immunity disorder in treating SLE by preventing the expression of Th1 type cytokines. The pathogenesis of SLE is complex and confusing, involving interactions between environmental and various aspects of the immune system. Serum IL-10 levels have been found higher in SLE patients and there is a statistically significant association of serum IL-10 with disease activity, with higher levels in active compared to inactive disease[25]. Moreover, it has been demonstrated that IL-10 levels are strongly implicated in the pathogenesis of SLE[26-28]. The result of this study has indicated that triptolide could decrease significantly the IL-10 level compared with that of model control group (Fig. 1b).

Systemic lupus often affects the kidney, excess of IgG and IgM deposition in the kidneys play a key role in the pathogenesis of murine and human lupus nephritis[29,30]. The results of present study demonstrated that treatment with triptolide reduced kidney damage. Light microscopic examination of the kidney tissues showed protection against attenuating severity of glomerulonephritis correlates well with reduced ECM of the glomeruli and the number of infiltrating inflammatory cells and deposition of IgG and IgM in glomeruli revealed with fluorescence microscopic examination (Fig. 2, 3, 4). One recent study found that (5R)-5-Hydroxytriptolide has a therapeutic benefit for lupus nephritis via suppressing chemokine expression and inhibiting immune cell infiltration in kidneys of MRL/lpr mice[31]. Our results are similar with the finding of previous study. In addition, accumulating evidence shows that Toll-like receptors (TLRs) including TLR4 and TLR9 upregulation at the protein or gene level are potent trigger to induce SLE. Therefore, TLRs and its downstream signal molecules (such as MyD88, NF-κB) are identified as potential therapeutic targets for SLE treatment[32-36]. In this study, it was found the mRNA levels of TLR9, TLR4 and NF-κB in kidney of MRL/lpr mice were significantly downregulated in triptolide-treated mice compared with model control group mice administered normal saline (Fig. 5). Previous results suggested that triptolide could suppress TLRs and related downstream signaling pathways in different diseases or cell models[37-39]. Present study results are similar with the findings of previous studies. Further studies are required to determine whether or not triptolide inhibits the signaling pathway of TLRs/NF-κB or other signaling pathways to alleviate systemic lupus symptoms in MRL/lpr mice.

CONCLUSION

The results showed that the triptolide derived from *Tripterygium wilfordii* Hook F. ameliorated skin damage, suppressed the serum levels of IFN-γ and IL-10 in MRL/lpr mice. In addition, triptolide could improve renal histopathologic characteristics and downregulate the mRNA level of TLR9, TLR4 and NF-κB in MRL/lpr mice. In other words, triptolide has the ability to improve the symptom of MRL/lpr mice. Thus, triptolide might play a role in humans as a novel strategy in the treatment of SLE.

SIGNIFICANCE STATEMENT

This study discovers the possible therapeutic utility of triptolide on lupus-prone MRL/lpr mice. Thus, it may represent a novel therapeutic strategy in the treatment of SLE. Moreover, this study will help the researcher to know more about the pharmacological effects of triptolide.

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