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Triptolide regulates the balance of Tfr/Tfh in lupus mice

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Abstract

Introduction/objectives Systemic lupus erythematosus (SLE) is a classic prototype of the multisystem autoimmune disease and follows a relapsing and remitting course. Triptolide is a diterpene triepoxide extracted from Chinese medicine Tripterygium wilfordii Hook F, with potent immunosuppressive and anti-inflammatory properties. Our previous work observed that triptolide alleviated lupus in MRL/lpr lupus mice with the upregulation of regulatory T cells (Treg) proportion in previous study. In this study, we explored the proportion of follicular T regulatory (Tfr), follicular T helper (Tfh) and germinal center (GC) B cells in lupus mice and evaluated the efficacy of triptolide for lupus treatment in *vivo*.

Methods 20 female MRL/lpr mice were randomly divided into 2 treatment groups and treated orally with vehicle or triptolide. C3H mice were all housed as controlled group and treated orally with vehicle. The percentage of Tfr cells, Tfh cells and GC B cells in spleen of mice were detected by Flow cytometric analysis and immunohistochemistry after 13 weeks of treatment.

Results We found that the percentage of Tfr cells decreased in MRL/lpr mice compared with controlled mice. The percentage of Tfh cells in MRL/lpr mice was significantly higher compared with that in controlled mice. The ratio of Tfr/Tfh is also decreased in lupus mice. After treated with triptolide in MRL/Lpr mice in *vivo*, the percentage of Tfr cells and ratio of Tfr/Tfh increased. The proportion of GC B cells also decreased in mice treated with triptolide by FACS and immunohistochemistry.

Conclusions Our results demonstrate that the effect of triptolide in alleviating lupus is partly by reversing immune imbalance with increased percentage of Tfr cells and ratio of Tfr/Tfh. Triptolide might also has effect on immune response through inhibiting proliferating GC B cells.

Keywords Triptolide, Systemic lupus erythematosus, Follicular T regulatory cell, Follicular T helper cell, Germinal center B cell

Introduction

Systemic lupus erythematosus (SLE) is a complex systemic autoimmune disease involving destruction in multiple organs and tissue inflammation [1]. Abnormal B cell activation and differentiation to memory or plasma effector cells secreting pathogenic autoantibodies play an important role in the humoral immune response in SLE. Autoantibodies produced by B lymphocytes and immune complex deposition in vital organs contribute to tissue damage primarily in the germinal centers (GCs), which forms in secondary lymphoid tissues. SLE may develop as a result of enhanced GC activity[2, 3]. The GC reaction

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is responsible for the generation of high affinity antibodies and long-lived plasma cells and is a highly regulated process. The dysregulation of mechanisms controlling GC reactions to exogenous or endogenous antigens may contribute to the emergence of SLE [4].

T follicular helper (Tfh) cells is essential for GC formation and B-cell function [5], which is characterized by a high expression level of CXC chemokine receptor 5 (CXCR5). CXCR5 was found to be indispensable for B-cell homing to B-cell follicles and T-cell migration to B-cell follicles [6]. Follicular regulatory T (Tfr) cells, a specialized subset of Treg cells, suppress the GC reaction after immunization and play an opposing role with Tfh cells in the regulation of humoral immunity [7, 8]. This population of cells shares common characteristics with Tfh and conventional Treg cells characterized by the expression of the master transcription CXCR5 and Foxp3. Uncontrolled Tfh or Tfr activity can result in the loss of immune tolerance and abnormal production of high levels of auto-antibodies, which can contribute to the development of autoimmune responses [9]. Current studies have revealed that imbalance of Tfh and Tfr subsets are indispensable for the balance between immune activation and tolerance, and may contribute to the pathogenesis of SLE by the regulation of GC responses and autoimmune responses [6, 10].

Tripterygium Wilfordii Hook F (TWHF) has been used in traditional Chinese medicine for centuries, mainly to treat a variety of autoimmune and inflammatory diseases as a immunosuppressant [11]. Triptolide, a diterpene triepoxide extracted from Tripterygium wilfordii Hook F, is one of the main bioactive ingredients, which has also been proven to have significant anti-inflammatory, immunosuppressive, anticancer and other important biological activities [12]. Our previous study demonstrated that triptolide upregulated the proportion of regulatory T cells (Treg) in MRL/lpr lupus mice [13]. T lymphocytes are increasingly being recognized as key contributors to SLE pathogenesis. Tfh cells enable autoantibody production, while defects in Treg cells lead to unchecked immune responses. Tfr cells are the Treg subset mainly localized in GCs and can mediate Tfh and B cell reactions for proper GC responses. It is unknown whether the effect of triptolide is related to Tfh cells or Tfr cells in lupus.

Triptolide has the effects of anti-inflammation and inhibition of cellular and humoral immunity. At the molecular level, triptolide is a global transcriptional inhibitor of a number of unrelated transcription factors including nuclear factor-kB (NF-kB), nuclear factor of activated T-cells (NF-AT), activator protein-1(AP-1) and heat shock transcription factor 1 (HSF1) [14]. The inhibitory effect of triptolide on transcription has been further verified through IL-1, IL-2, IL-4, IL-6, TNF-a, IFN-a, CD40, CD80 and other proteins that play key roles in inflammation and immunoregulation [15]. At the humoral immunity level, the extract of Tripterygium wilfordii hook F inhibited the production of the immunoglobulins IgM, IgG and IgA by stimulated B-lymphocytes [16]. We hypothesized that the effect of triptolide on humoral immunity is related to Tfr or Tfh cells.

In this study, we tested Tfh cells, Tfr cells and the ratio of Tfr/Tfh in MRL/lpr lupus mice. We found that Tfr cells and the ratio of Tfr/Tfh in MRL/lpr lupus mice is decreased compared with controlled mice. We investigated the therapeutic effects of triptolide on MRL/lpr lupus mice in *vivo*. After treated with triptolide, Tfr cells and the ratio of Tfr/Tfh in MRL/lpr lupus mice increased. Triptolide also decreased the GC B cells in *vivo*. The alterations in Tfh and Tfr cells may contribute to dysregulated immunity and the pathogenesis of SLE. Our results suggested that triptolide alleviate lupus disease possibly by reversing the Tfr/Tfh imbalance and decreasing splenic frequencies of GC B in lupus mice.

Materials and methods

Reagents

Triptolide was purchased from Sigma (St. Louis, MO), which were ≥ 98% pure, as assessed by High Performance Liquid Chromatography (HPLC). Urine protein test kit were purchased from Nanjing Jiancheng Bio-engineering institute (Nanjing, China). All antibodies were purchased from Miltenyi Biotec or eBioscience. Data were collected with a fluorescence activated cell sorting (FACS) Calibur flow cytometer (BD Biosciences) and analyzed by CellQuest software (BD Biosciences).

Mice

Seven-week-old female MRL/lpr mice and C3H wild-type mice were obtained from Shanghai SJA laboratory Co., Ltd. (Shanghai, P.R.China). All mice were housed under specific pathogen-free and housing conditions and kept in a 12/12 h light and dark cycle and free intake of feed and water. 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.

Experimental design

In our previous study, MRL/lpr mice were treated orally with triptolide 0.2 mg/kg/d and triptolide 0.3 mg/kg/d and the proteinuria levels of mice were measured every two weeks until 13 weeks of treatment [13]. According to the previous results, we chose the best concentration of triptolide with no death after 13 weeks of treatment.

For therapy, 20 female MRL/lpr mice at 7 weeks of age were randomly divided into 2 treatment groups were all treated orally with vehicle (1% DMSO/1% Tween 20 in ddH_2O, n = 10) or 0.2 mg/kg/d triptolide (n = 10) after one week adaptation to the environment. Seven-week-old mice (C3H, n = 6) were all housed as controlled group and treated orally with vehicle. 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.

ELISA

The urinary protein was quantitatively detected using urine protein test kit (KeyGen BioTECH, Nanjing, Jiangsu, China) according to the manufacturer's instructions. Anti-dsDNA Abs in the mice serum were determined by ELISA. ELISA plates (Costar, Cambridge, MA) were pretreated with protamine sulfate (Sigma, St. Louis, MO) and then coated with 50 mg/ml calf thymus dsDNA (Sigma, St. Louis, MO). 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).

Histological analysis

The kidneys were fixed in 4% paraformaldehyde (PFA) for 24 h and embedded in paraffin. Three μm sections were prepared for hematoxylin and eosin staining (H&E; Sinopharm Chemical Reagent, Shanghai, P.R. China). The sections were scored by two professional renal pathologists, who have examined the tissues for glomerular, interstitial, and vascular lesions according to the previously reported criteria in a blinded manner. In brief, a 0–4 + scale was used to grade glomerular, interstitial, and vascular according to specified criteria of pathology as absent, mild, moderate, or severe. We evaluated renal pathology using coded slides.

Isolation mice splenocytes

Freshly prepared spleens 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 Phosphate Buffer Solution(PBS).

Flow cytometric analysis

Mice splenocytes were stained for surface markers with mouse Percp cy5.5-anti-CD4(clone RM4-5), APC-anti-CXCR5(clone 2G8), efluor 660-anti-GL-7(GL-7), FITC-anti-B220(clone RA3-6B2) and then the cells were fixed and permeabilized with Cytfix/Cytoperm (eBioscience) and stained with FITC-anti-Foxp3(3G3). All antibodies were purchased from BD Biosciences or eBioscience. Flow cytometric analysis was performed on a FACS Calibur (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.

Statistical analysis

Quantitative data were expressed as mean ± standard deviation and analyzed with Prism 9 (GraphPad Software). Differences determined by two-tailed Student' s *t*-test were used for two-group comparisons. ANOVA followed by Bonferroni post-hoc test was used for multiple comparisons. All P values < 0.05 were considered significant.

Results

The percentage of tfr cells and Tfr/Tfh ratio decreased in spleen of MRL/lpr mice

MRL/lpr mice develop a syndrome that is serologically and pathologically similar to human systemic lupus erythematosus (SLE). In our study, we compared Tfh and Tfr cells in twenty-week-old female MRL/lpr mice and C3H wild-type mice. The proportion of $Foxp3^+CXCR5^+CD4^+$ Tfr cells in spleen of MRL/lpr mice was significantly lower compared with that in wild-type controlled mice (C3H mice) (1.265 ± 1.238% in controlled group vs. 0.2229 ± 0.1549% in MRL/lpr group). (Fig. 1A and B), while the percentage of $Foxp3^-CXCR5^+CD4^+$Tfh cells in spleen of MRL/lpr mice was significantly higher in spleen compared with that in wild-type controlled mice (0.5333 ± 0.8543% in controlled group vs. 3.184 ± 0.2685% in MRL/lpr group). (Fig. 1C). The Tfr/Tfh ratio in spleen of MRL/lpr mice was significantly lower compared with that in controlled mice (7.079 ± 5.197% in controlled group vs. 0.08484 ± 0.06017% in MRL/lpr group) (Fig. 1D).

Triptolide treatment relieved proteinuria, anti-dsDNA IgG autoantibodies and histological symptoms in MRL/lpr mice

In order to assess the effect of triptolide therapy on lupus symptoms of MRL/lpr mice, we analyzed the kidneys of the mice treated with triptolide. After 13 weeks of treatment, two MRL/lpr mice died of disease in vehicle-treated mice group while there was no death in triptolide-treated mice. We found that kidney lesions of lupus mice and the infiltration of inflammatory cells were reduced by triptolide treatment (Fig. 2A, B). Besides, the

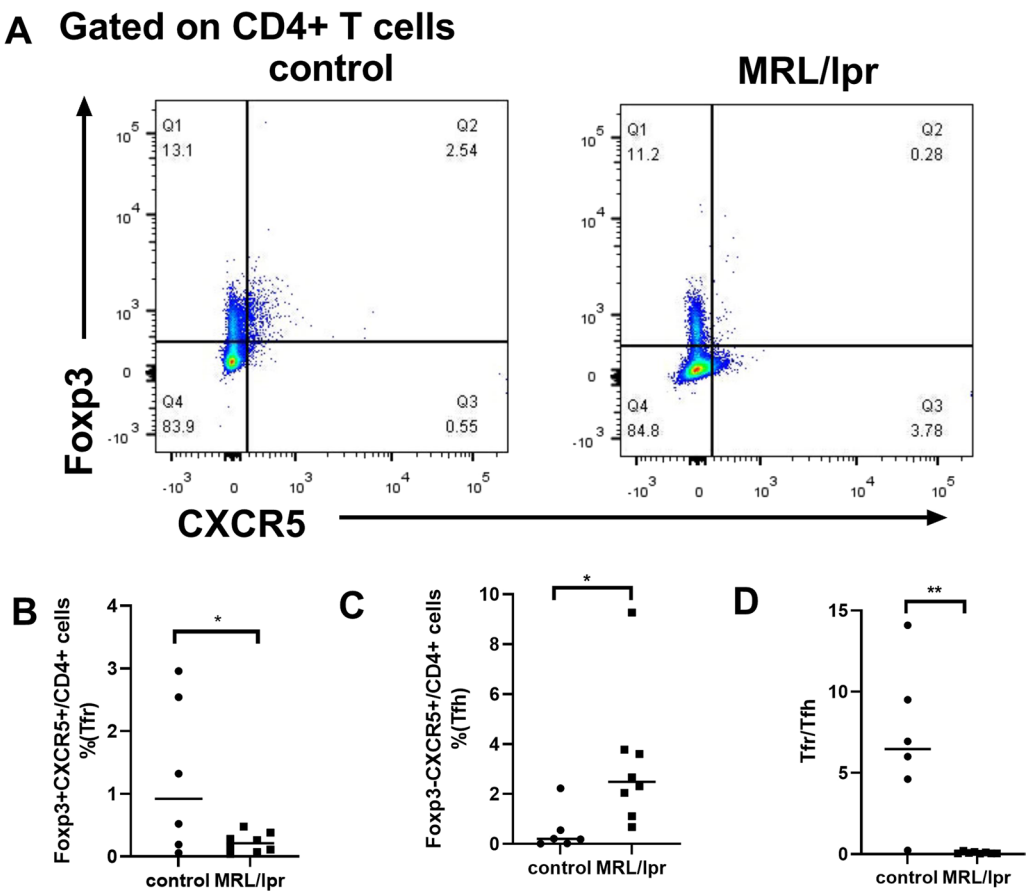

Fig. 1 Comparisons of frequencies of Tfr cells, Tfh cells and Tfr/Tfh ratio in MRL/lpr mice (n = 8) and controlled mice (C3H) (n = 6). **A** Representative staining of Tfr ($CD4^+Foxp3^+CXCR5^+$) cells and Tfh ($CD4^+Foxp3^-CXCR5^+$) among $CD4^+$ T splenocytes in MRL/lpr lupus mice and controlled mice (C3H). **B** The percentage of $CD4^+Foxp3^+CXCR5^+$ Tfr cells in spleen of MRL/lpr mice was significantly lower than that in controlled mice (C3H). **C** The percentage of $CD4^+Foxp3^-CXCR5^+$Tfh cells in spleen of MRL/lpr mice was significantly higher than controlled mice (C3H). **D** The ratio of Tfr/Tfh in spleen of MRL/lpr mice was significantly lower than that in controlled mice (C3H) *$p < 0.05$, ** $p < 0.01$

urine protein and serum anti-dsDNA antibody were also decreased after treated with triptolide (Fig. 2C, D). All these data indicated that triptolide ameliorated MRL/lpr mice lupus disease.

Triptolide treatment increased the proportion of Tfr and Tfr/Tfh ratio in MRL/lpr mice

In previous studies [13], Tfh cells are increased in the peripheral circulation of SLE patients and correlate with SLE severity, and inhibition of Tfh cells might reduce autoantibody production during the treat of SLE. The ratio of circulating Tfh cells to Tfr cells is correlated with disease activity in SLE patients. In our study, as shown in Fig. 3A and B, the proportion of $CD4^+CXCR5^+Foxp3^+$Tfr subset increased significantly in the triptolide-treated MRL/lpr mice compared with vehicle-treated MRL/lpr mice (0.2229 ± 0.1549% in vehicle group vs. 0.5322 ± 0.2133% in triptolide group). As shown in Fig. 3C, the proportion of $CD4^+CXCR5^+Foxp3^-$Tfh showed no significant difference in the spleen of triptolide-treated MRL/lpr mice compared with vehicle-treated MRL/lpr mice (3.184 ± 2.685% in vehicle group vs. 3.772 ± 2.421% in triptolide group). As shown in Fig. 3D, the ratio of Tfr/Tfh in spleen were significantly increased. These data suggest that triptolide enhanced the proportion of Tfr cell and the Tfr/Tfh ratio in MRL/lpr mice.

Triptolide treatment decreased the proportion of GC B in MRL/lpr mice

B cells rapidly expand and differentiate, and then generate plasma cells that produce high affinity antibodies after activation by antigen in GCs [17]. The process is critically dependent on the intimate interaction of GC B cells and Tfh cells. In our study, we detect GL-7^+B220^+ cells in MRL/lpr mice after treated with triptolide. As shown in Fig. 4A and B, the percentage of GL-7^+B220^+ GC B cells

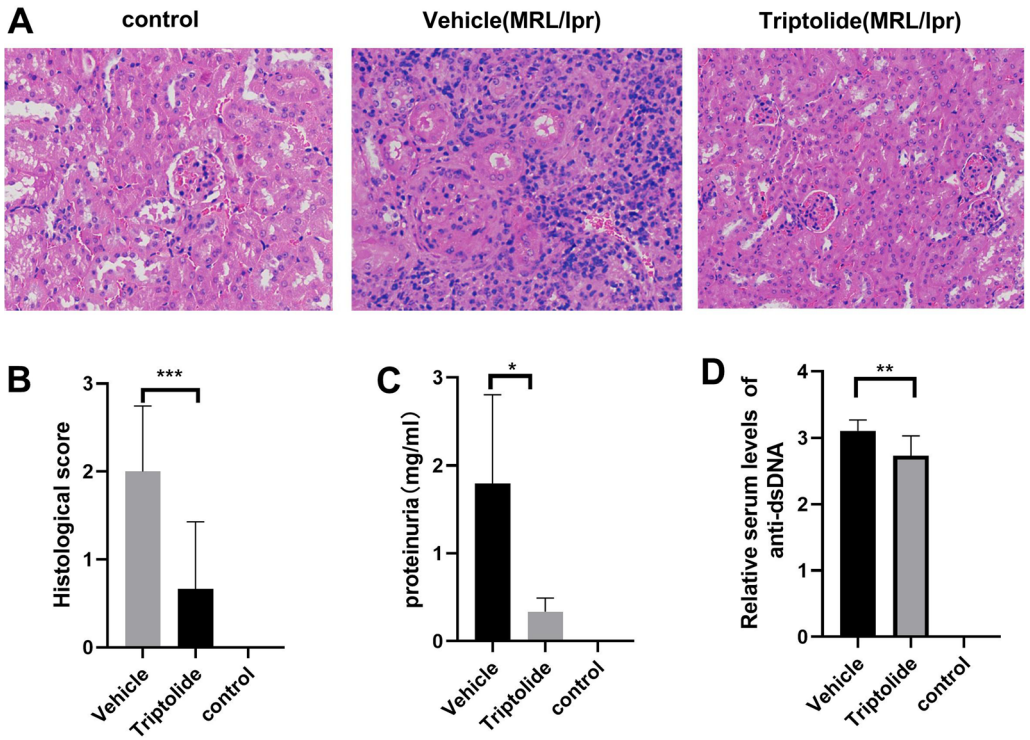

Fig. 2 Triptolide ameliorated lupus nephritis of MRL/lpr mice. **A** Representative kidney sections stained with H&E (×100). **B** Histological score for glomerulonephritis, interstitial nephritis and tubular casts, according to H&E staining in MRL/lpr lupus mice (n = 8) and controlled mice(C3H) (n = 6). **C** The proteinuria levels in two groups were measured at the end of study. **D** The anti-dsDNA IgG autoantibodies in two groups were measured at the end of study. *$p < 0.05$; ***$p < 0.001$

in spleen of MRL/lpr mice was significantly higher than that in controlled mice (1.695 ± 0.2288% in controlled group vs. 3.472 ± 1.129% in MRL/lpr group). After treated with triptolide, the proportion of GL-7^{+}B220^{+} GC B cells in spleen of MRL/lpr mice was significantly decreased. These data suggest that triptolide decreased the proportion of GC B cells in MRL/lpr mice (3.472 ± 1.129% in vehicle group vs. 2.279 ± 0.5116% in triptolide group). Next, we examined the GCs by immunohistochemistry of frozen spleen sections from the MRL/lpr mice treated with triptolide and vehicle. As shown in Fig. 4C, there were many distinct GCs in the spleens of vehicle-treated MRL/lpr mice as revealed by peanut agglutinin (PNA) and anti-IgD staining. In contrast, GC B cells were significantly decreased in triptolide treated mice. Thus, triptolide treatment could also suppress the GC response in MRL/lpr mice.

Discussion

In this study we revealed for the first time that triptolide could enhance the percentage of Tfr cells and the ratio of Tfr/Tfh in MRL/Lpr mice in vivo. GC B cells in spleens were decreased in triptolide-treated mice by FACS and immunohistochemistry.

CD4^{+} T cells have a crucial role in helping or inhibiting B cells to produce Ab and can differentiate into various subsets of helper T (Th) cells such as Treg cells, Tfh cells, Tfr cells, etc. In our previous study [13], triptolide enhanced the proportion of Treg cells in lupus-prone MRL/lpr mice. Considering that traditional Chinese medicine views the human body as a complex dynamical system, and focuses on the balance of the human body, we examined the therapeutic effect of triptolide in lupus mice and whether it related Tfh cells and Tfr cells. Our results showed that triptolide-treated MRL/lpr mice have significantly decreased proteinuria and lupus nephritis evidenced by renal histopathologic assessment, compared with vehicle-treated mice. MRL/lpr lupus mice displayed an increased percentage of Tfr cells and ratio of Tfh/Tfr compared with controlled mice.

Different studies have demonstrated different results of numbers of Tfr cells and Tfr/Tfh ratio in SLE patients. Tfr cells in new onset SLE patients has been reported to be lower than those in the healthy controls[18], while other study has reported that the frequency of circulating Tfr cells decreased and Tfh/Tfr ratio increased in SLE patients [19]. The different results may be attributed to different strategies employed to select SLE patients.

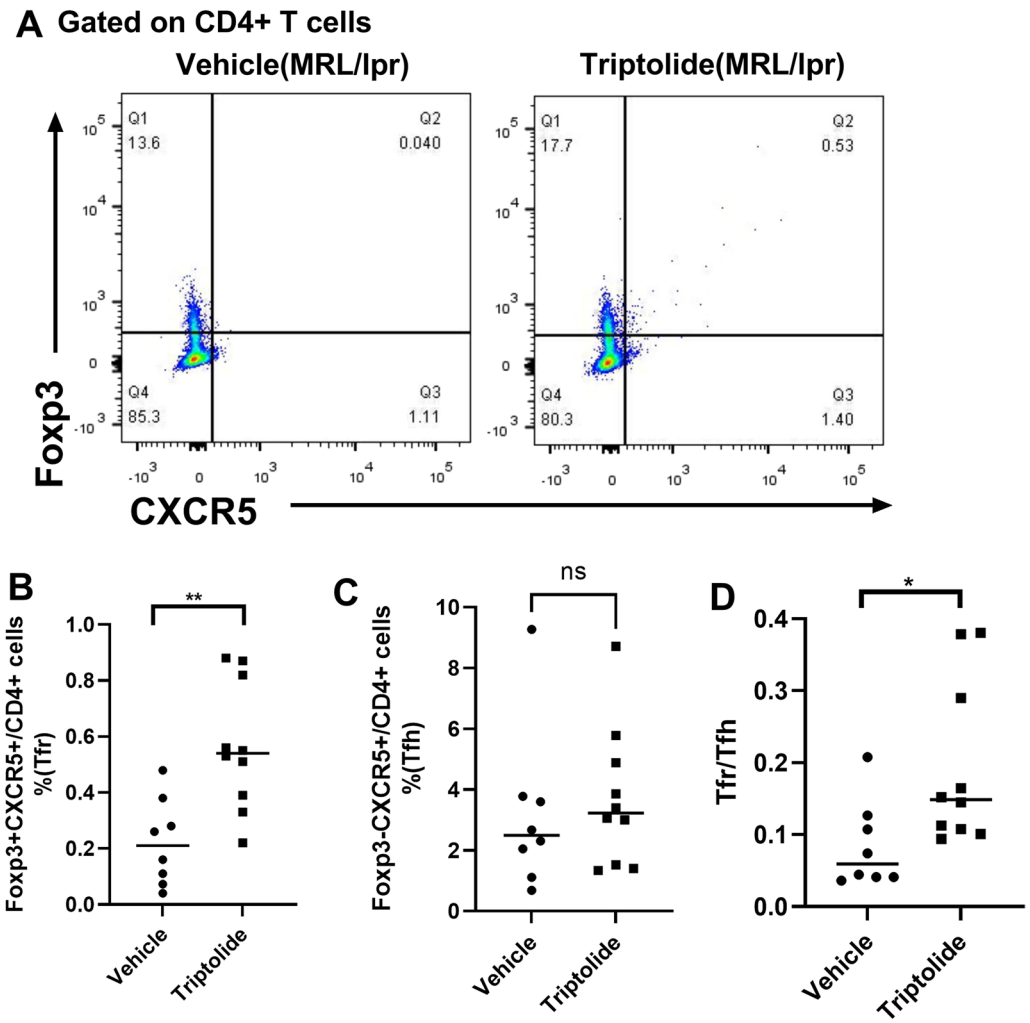

Fig. 3 Triptolide increased Foxp3$^+$CXCR5$^+$Tfr and Tfr/Tfh in MRL/lpr mice. **A** Representative staining of Tfr (CD4$^+$Foxp3$^+$CXCR5+) cells and Tfh (CD4$^+$Foxp3$^-$CXCR5$^+$) among CD4$^+$ T splenocytes in triptolide-treated MRL/lpr mice and vehicle-treated MRL/lpr mice. **B** The percentage of CD4$^+$Foxp3$^+$CXCR5$^+$ Tfr cells in spleen of triptolide-treated MRL/lpr mice (n = 10) was significantly higher than that in vehicle-treated MRL/lpr mice (n = 8). **C** The percentage of CD4$^+$Foxp3$^-$CXCR5$^+$ Tfh cells in spleen of two groups of mice showed no difference. **D** The ratio of Tfr and Tfh in spleen of triptolide-treated MRL/lpr mice was significantly higher than that in vehicle-treated MRL/lpr mice. *$p < 0.05$; ** $p < 0.01$

MRL/lpr mice, one of the representative animal models of lupus, are produced by mating four strains of mice, namely LG, AKR, C3H, and C57BL/6, which are similar to systemic lupus erythematosus patients, such as abnormalities in T and B cells, enlarged lymph nodes, high titers of anti dsDNA and so on. C3H mice are often used as normal controls for MRL/lpr mice [20, 21]. In our study, MRL/lpr lupus mice displayed a significantly lower proportion of Foxp3$^+$CXCR5$^+$CD4$^+$ Tfr cells and significantly higher percentage of Foxp3$^-$CXCR5$^+$ CD4$^+$ Tfh cells in spleen compared with that in wild-type controlled mice (C3H mice). The Ratio of Tfr/Tfh in spleen of MRL/lpr mice was significantly lower compared with that in controlled mice. Tfh cells play a significant role in the auto-Ab responses in SLE [10]. Tfr cells inhibit Tfh cells and germinal center (GC) responses and have opposite roles in regulating humoral immunity. Therefore the balance of Tfh/Tfr cell is critically important for the maintenance of immune tolerance [22]. The frequency of Tfr and Tfh/Tfr ratio but not that of Tfh was correlated with diseases activity in SLE. In addition, increase in Tfr cell numbers and decrease in the Tfh/Tfr ratios were observed with successful treatments in human[19]. Our results suggested that the specific mechanism of Tfr cells in the pathogenesis of lupus mice can be further studied in the future.

There are still limitations in the present study. Tfh cells can be defined through the combination of

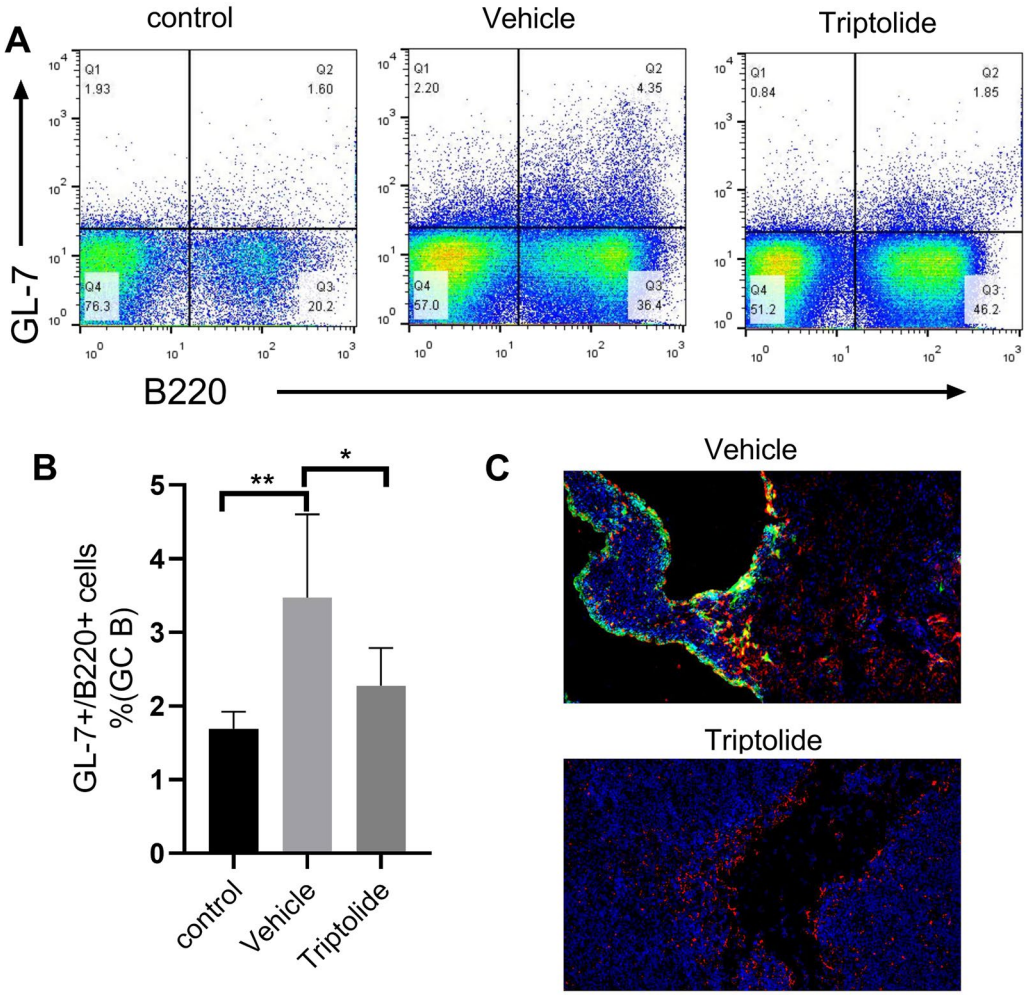

Fig. 4 Triptolide significantly upregulated GL-7$^+$B220$^+$ cells. **A** Representative staining of GC B cells(B220$^+$GL-7$^+$) among mice splenocytes by FACS. **B** The percentage of GL-7$^+$B220$^+$ GC B cells (n = 8) in spleen of MRL/lpr mice was significantly higher than that in controlled (C3H) mice (n = 6). The percentage of GL-7$^+$B220$^+$ GC B cells in spleen of triptolide-treated MRL/lpr mice (n = 10) was significantly lower than that in vehicle-treated MRL/lpr mice (n = 8). **C** Immunohistochemistry of GCs after triptolide treatment. Cryosection of spleens were stained with PNA (red) and anti-IgD (green) to show GCs in B cell follicles and imaged at 40x magnification. Representative images from four mice per treatment. *$p < 0.05$, ** $p < 0.01$

markers as follows: CXCR5, CD127 and IL-21, PD-1, and ICOS, etc. [9]. In our results, the proportion of CD4$^+$CXCR5$^+$Foxp3$^-$Tfh showed no significant difference in triptolide-treated MRL/lpr mice. More markers in Tfh cells of lupus mice could be detected in future. Then which Tfh subgroup can triptolide affect are still required to study to uncover the precise molecular mechanisms during the pathogenic process of lupus.

GCs are the site a specialized microstructure of producing long-lived antibody secreting plasma cells and memory B cells, which are vitally important for humoral immunity. Regulation of GC reactions is critical to ensure high affinity antibody production and to enforce self-tolerance by avoiding emergence of autoreactive B cell clones [17]. Many mice models of SLE are characterized by spontaneous GC development, which demonstrate the important role of the GC in the acquisition of autoreactivity in SLE [4].Targeting of the GC has been a major focus of therapying SLE.

Triptolide has been identified as one of the most potent immunosuppressive and anti-inflammatory compounds isolated from Tripterygium in in many studies [23]. Triptolide inhibits lymphocyte activation and T-cell expression of interleukin-2 at the level of transcription. But IL-2 is critically required for the homeostatic immune maintenance, as it stabilizes Foxp3 expression and maintains lineage identity of Treg [24]. Defective IL-2 production contributes to the unbalanced immune system in SLE [25]. Clinical trials also suggested that low-dose IL-2 might be effective in treatment of SLE [26]. How

triptolide plays a role in the treatment of lupus when it inhibiting IL-2. Our study for the first time demonstrated that triptolide treatment probably inhibit the activity of SLE by Tfr cells and the GC response through reducing GC B cells.

Our data showed that the percentage of GL-7^{+}B220^{+} GC B cells in spleen of MRL/lpr mice was significantly higher than that in controlled mice at the end of study. Previous reports showed that GC B cells (B220^{+}GL-7^{+} gated on $CD3^{-}$) typically decreased in number as the lupus-prone mice aged [27]. In our future research, we can further study the effect of triptolide on GC B cells of different age in MRL/lpr mice.

Regulation of GC responses is not a simple outcome of Tfr/Tfh balance, but also involves the contribution of other cell types to modulate the GC microenvironment and to avoid autoimmunity. In our study, triptolide treatment did not affect Tfh cells that are specialized T helper subset to help GC B cells. Therefore, further studies regarding the other cell types to modulate the GC microenvironment in triptolide treatment are necessary.

Triptolide, isolated from the traditional Chinese herb TWHF, is approved and widely used to treat many kinds of autoimmune diseases due to its favorable cost-benefit ratio and good efficacy [11]. A widespread application of triptolide raises the question on the safety of its use in clinical settings [28]. In our study, there is no death in mice treated with triptolide (0.2 mg/kg/d) in *vivo*. Further studies regarding the toxicity of triptolide concentration in clinical settings are necessary.

Conclusion

In summary, 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 triptolide may ameliorates lupus by inhibiting the activity and progress of SLE by increasing the percentage of Tfr cells and proportion of Tfr/Tfh, and inhibiting the GC response through decreasing GC B cells. The study added more evidence to support a clinical trial of triptolide in the therapeutic efficacy of SLE.

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Not applicable.

Authors' contributions
All authors participated in the design of the study. Statistical analyses were conducted by Xia Zhao and Feng Guo. All authors contributed to interpretation of the results. Xia Zhao contributed to the drafting of the manuscript. All authors contributed to the critical revision of the manuscript for important intellectual content, approved the final version and are accountable for the integrity of its content.

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

Declarations

Ethical approval and Consent to participate
Approved by the Institutional Animal Care and Research Advisory Committee.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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