International Immunopharmacology 74 (2019) 105677

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Cryptotanshinone ameliorates the pathogenesis of systemic lupus erythematosus by blocking T cell proliferation

Yu Du[1], Lijun Du[1], Zhixing He, Jia Zhou, Chengping Wen*, Yun Zhang*

Institute of Basic Research in Clinical Medicine, College of Basic Medical Science, Zhejiang Chinese Medical University, Hangzhou 310053, China

ARTICLE INFO

Keywords:
Cryptotanshinone
STAT3
Systemic lupus erythematosus
T cell proliferation

ABSTRACT

Systemic lupus erythematosus (SLE) is a chronic, devastating autoimmune disorder associated with severe organ damage. Recently, the role of Signal Transducer and Activator of Transcription 3 (STAT3) in murine lupus has been described, suggesting the involvement of STAT3 signaling in the development of SLE. Cryptotanshinone (CTS) is an effective inhibitor of STAT3; however its potential as a SLE treatment remains to be explored. To determine the function of CTS in SLE, we treated MRL/*lpr* female mice with CTS. Firstly, we found CTS treatment reversed the elevated STAT3 signaling of spleens in lupus-prone MRL/*lpr* mice, accompanying with a dramatically decreased number of T cells, especially double-negative (DN) T cells. Further research showed that CTS inhibited T cell proliferation via suppressing of STAT3 activation in vitro and in vivo. Consistently, we also proved that CTS treatment significantly alleviated autoimmune response including notably diminished skin lesions, reduced spleen size and increased life span. In addition, CTS treatment decreased the levels of autoantibodies and pro-inflammatory cytokines, as well as normalized structure and function of kidneys. All these data suggested that CTS treatment depressed STAT3 phosphorylation, which resulted in blocked DN T cell proliferation and finally attenuated the spontaneous SLE development. Taken together, our data identify CTS as a potential therapeutic drug for SLE patients.

1. Introduction

Systemic lupus erythematosus (SLE), a systemic autoimmune disease, results from aberrant activation of both the innate and adaptive immune systems [1] and is characterized by the accumulation of autoantibodies and deposition of immune complexes in various organs, particularly the kidneys [2]. Dysfunctional T cell activation significantly contributes to SLE pathogenesis in three aspects including providing help to auto-reactive B cells, producing inflammatory cytokines and infiltrating target-organs [3,4]. Although an improved understanding of the mechanisms involved in the pathogenesis of SLE has offered the foundation for novel treatments [5], the development of new therapies for SLE is necessary due to the heterogeneity of this disease.

Signal Transducer and Activator of Transcription 3 (STAT3) plays an important role in the host inflammatory response and innate/adaptive immune response [6]. STAT3 is activated by kinase-mediated phosphorylation at Tyr705 [7] and two phosphorylated STAT3 proteins form a homodimeric-activated transcription factor complex. Subsequently, the complex translocation to the nucleus induces target gene expression [8]. Accumulating evidence suggests that STAT3 is involved in SLE development and inhibition of STAT3 activation delays the onset of lupus nephritis. Thus the STAT3 pathway represents a promising novel treatment for SLE [3,9]. Cryptotanshinone (CTS), a major active quinoid diterpene in dried roots of the Chinese herbal medicine Salvia miltiorrhiza Bunge (Danshen), has been demonstrated to be an effective inhibitor of STAT3 [10,11]. In the current study, we examined the effect of CTS as a new therapeutic agent for SLE treatment via targeting STAT3 signaling.

MRL/*lpr* mice, carrying the *lpr* mutation in the *Fas* gene, which causes a deficit in Fas-mediated apoptosis of T cells and exaggerated activation of B cells [12], spontaneously develop syndromes resembling human SLE and are regarded as good surrogates for studying this disease [13]. To test our hypothesis, we treated female MRL/*lpr* mice with vehicle control or CTS from 12 weeks to 20 weeks and found that CTS-treated MRL/*lpr* mice showed decreased STAT3 phosphorylation, accompanied with the reduction accumulation of T cells, especially DN T cells in spleens. Further research showed that CTS inhibited DN T cell proliferation via suppressing STAT3 activation in vitro and in vivo, finally resulting in reduced T cell accumulation. Consistently, we found

* Corresponding authors at: College of Basic Medical Science, Zhejiang Chinese Medical University, 548 Binwen Rd., 310053 Hangzhou, China.
E-mail addresses: chengpw2010@126.com (C. Wen), zhangyun@zcmu.edu.cn (Y. Zhang).
[1] The authors contribute equally to this work and are assigned as co-first authors.

https://doi.org/10.1016/j.intimp.2019.105677
Received 1 April 2019; Received in revised form 30 May 2019; Accepted 31 May 2019
Available online 07 June 2019

CTS treatment alleviated lupus-like syndrome including reduced onset of autoimmunity, decreased production of auto-antibodies, mitigated lupus nephritis and improved kidney function. As a result, we identified CTS as a key inhibitor of STAT3 pathway, thereby inhibited DN T cell proliferation and contributed to the amelioration of SLE. Our research suggests that CTS may be used as a novel therapeutic strategy for SLE treatment.

2. Materials and methods

2.1. Mice

MRL/*lpr* and MRL/MpJ female mice (4–5 weeks) were purchased from SLRC Experimental Animals Co. Ltd. (Shanghai, China). Cryptotanshinone (CTS) (≥98%, HPLC) was purchased from Sigma-Aldrich (St. Louis, MO, USA). From 12 weeks, MRL/*lpr* or MRL/MpJ mice were randomly divided into three groups and administered with vehicle control, CTS 10 mg/kg or 30 mg/kg via gastric gavage every day for 9 weeks. The deaths were recorded every day. At 20 weeks, the mice were sacrificed, skin lesions were photographed, and then the spleens and kidneys were collected. The WBCs (white blood cells) of peripheral blood and spleen cells were counted. All animal experiments in this study were reviewed and approved by the Institutional Animal Care and Use Committee of Zhejiang Chinese Medical University.

2.2. Renal histology and immunofluorescence (IF)

Kidneys were harvested from exsanguinated mice, immediately fixed with 4% formalin and then embedded in paraffin according to standard procedures. Sections (5 μm) were mounted on slides for hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) staining to evaluate the morphology changes and inflammation level in kidneys. Renal pathology was scored by an experienced pathologist blinded to the treatments in accordance with previous studies [14,15]. In brief, glomerular deposits, glomerular crescent formation (glomerular indices), interstitial inflammation, endocapillary proliferation and tubular casts and dilatation (tubulointerstitial indices) were each scored from 0 to 4, with 0 meaning normal, 1 or 2 representing a slight hyperplasia in the glomerular mesangium, 3 indicating glomerular lobular formation and thickened basement membrane and 4 indicating glomerular crescent formation, tubular atrophy and sclerosis. The score of each mouse was calculated from the total scores of observed five glomeruli. In addition, immune complex (IgG) deposition was detected with IgG-FITC antibody (Abcam Ltd., Cambridge, MA, USA) according to a previous protocol [16] and the fluorescence intensity was quantified by Image J.

2.3. Urinalysis

Urine was manually collected from each mouse daily from 19 weeks. Fresh urine samples were centrifuged at 1500 rpm for 10 min at 4 °C and pooled for each mouse and then stored at −80 °C until use. Levels of total protein, albumin and creatine in urine were assessed using commercially available kits obtained from Dia Sys Diagnostic Systems GmbH (Holzheim, Germany).

2.4. ELISA

Serum samples were collected from whole blood without anticoagulants at 3000 rpm for 10 min at 4 °C. The concentrations of anti-dsDNA (SHIBAYAGI Co., Ltd., Shibukawa, Japan), IgG and IFN-γ (Multisciences Biotech Co., Ltd., Shanghai, China) in serum were determined with corresponding ELISA kits according to the manufacturer's instructions.

Table 1
Primers used for real-time PCR in our study.

Genes	Primers(5′-3′)
actin	GGCTGTATTCCCCTCCATCG
	CCAGTTGGTAACAATGCCATGT
ifn-α	GCTAGGCTCTGTGCTTTCCT
	GGCTCTCTTGTTCCTGAGGT
ifn-β	AGCTCCAAGAAAGGACGAACA
	GCCCTGTAGGTGAGGTTGAT
ifn-γ	ATGAACGCTACACACTGCATC
	CCATCCTTTTGCCAGTTCCTC
ccl2	TTAAAAACCTGGATCGGAACCAA
	GCATTAGCTTCAGATTTACGGGT
il-6	TAGTCCTTCCTACCCCAATTTCC
	TTGGTCCTTAGCCACTCCTTC
il-17a	TTTAACTCCCTTGGCGCAAAA
	TTTCCCTCCGCATTGACAC

2.5. RT-PCR and real-time PCR

Total RNA from kidneys was extracted using TRIzol (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The concentration of RNA was measured with a Nanodrop spectrophotometer (Thermo Fisher Scientific). Total RNA (2 μg) was reverse-transcribed using the ReverTra Ace® qPCR RT Kit (Toyobo, Osaka, Japan). Real-time PCR was performed using the Fast Start Universal SYBR Green Master Kit (Roche, Mannheim, Germany) and 7900HT Fast Real-Time PCR System (Thermo Fisher Scientific). The following PCR primer sequences were obtained from previous study [14] and PrimerBank (https://pga.mgh.harvard.edu/primerbank/). The primers used are listed in Table 1. The comparative C_t ($2^{-\triangle\triangle Ct}$) method was used to quantify PCR data.

2.6. FACS analysis

To measure T/B cell percentage, red blood cells were removed from peripheral blood, and single-cell suspensions of spleens were stained with PE-anti-CD3- and FITC-anti-CD19 antibodies (Thermo Fisher Scientific) followed by FACS analysis using an FC 500 MC system (Beckman Coulter, Fullerton, USA). FITC-anti-CD3, PE-anti-CD4 and APC-anti-CD8 antibodies (Thermo Fisher Scientific) were used to analyze the T cell subsets in spleens.

2.7. Purification of T cells and DN T cells

Total T cells isolated from spleens of MRL/*lpr* and MRL/MpJ mice were purified with negative selection using a Mouse T Cell Isolation Kit (Stem Cell Technologies Inc., Vancouver, Canada). In brief, 50 μl of rat serum was added to cell samples per 1×10^8 cells in 1 ml PBS containing 2% FBS and 1 mM EDTA. Then cell samples were transferred to the requisite tubes. 50 μl of isolation was added to the samples, and the samples were cocktail, mixed and incubated for 10 min. After vortexing streptavidin RapidSpheres™ for 30 s, 75 μl of the RapidSphere suspension was mixed with each sample and incubated for 2.5 min. Subsequently, the samples were added to 2.5 ml of PBS containing 2% FBS and 1 mM EDTA and mixed. Finally, the tubes were placed into the magnet and incubated for 5 min. All stages of the protocol were performed at room temperature and the enriched cell suspension was purified T cells. On this basis, DN T cells were isolated with Fluorescent cell sorting with anti-CD3, anti-CD4 and anti-CD8 antibodies using BD FACSAria.

2.8. Immunoblotting analysis

Total splenocytes or purified T cells obtained from MRL/*lpr* and MRL/MpJ mice as well as DN T cells isolated from MRL/*lpr* mice were

homogenized in RIPA buffer (Beyotime Biotechnology, Shanghai, China) and supplemented with protease and phosphatase inhibitors (Beyotime Biotechnology) before further analysis. Cell lysates (40 μg) were separated on SDS-PAGE and immunoblotted using antibodies against the following proteins: phospho-STAT3 (Y705), STAT3 (Abcam Ltd) and actin (Sigma-Aldrich).

2.9. T cell proliferation assay

Purified total T cells or DN T cells were resuspended in complete medium (RPMI 1640 with 10% heat-inactivated FBS, 50 U/ml of penicillin and 50 U/ml of streptomycin). Then 1×10^5 cells, in a volume of 100 μl per well, were cultured in a 96-well plate, with or without incubation with 1 μg/ml anti-CD3 antibody (Biolegend, San Diego, CA, USA) and 0.5 μg/ml anti-CD28 antibody (Biolegend) to activate naïve T cells. Plated cells were cultured for 48 h in the presence of vehicle control or CTS (10 μM) and the T cell number was assessed by a Cedex XS cell analysis system (Roche).

2.10. Statistical analysis

Data are expressed as the mean ± SE and were assessed as significance by *t*-test or two-way ANOVA using Graphpad Prism 8, as appropriate. If ANOVA was significant, individual differences were determined with Tukey post-test. In our studies, p values < 0.05 were considered significant.

3. Results

3.1. CTS treatment reverses the increased STAT3 activation in lupus-prone MRL/lpr mice

MRL/*lpr* mice are genetically predisposed to spontaneously develop an autoimmune syndrome that is similar to human SLE in several key features, such as the production of various auto-antibodies, immune complex accumulation, splenomegaly and development of glomerulonephritis [17]. Symptoms of SLE develop from 12 weeks onward in MRL/*lpr* mice [18]. As a result, we treated MRL/*lpr* and MRL/MpJ female mice with either vehicle control or CTS (10 mg/kg and 30 mg/kg) every day for a period of 9 weeks, beginning at 12 weeks of age (Fig. 1A). STAT3 signaling, the main target of CTS, has been demonstrated to be involved in SLE development. As a result, we firstly measured STAT3 phosphorylation in spleen lysates obtained from 20-week-old MRL/*lpr* and MRL/MpJ mice and results showed that CTS normalized the improved STAT3 phosphorylation of lupus mice dose-dependently (Fig. 1B–C), which suggested that CTS were effective in lupus mice.

3.2. CTS treatment reduces DN T cell number in MRL/lpr spleens

One of the hallmarks in lupus is the over-accumulation of T cells. We thus want to know whether CTS can influence T cell number to regulate the immune response in SLE. We isolated the whole splenocytes from vehicle- or CTS-treated MRL/*lpr* and MRL/MpJ mice and found a decreased number of splenocytes in MRL/*lpr* mice (Fig. 2A) in response to CTS. Further analysis of immune cells obtained from spleens demonstrated that CTS observably inhibited the T cell number (Fig. 2B) while slightly increased the number of B cells (Supplemental Fig. 1), which suggested that the use of CTS may trigger a feedback mechanism to promote the differentiation or proliferation of B cells. Then in-depth research showed that CTS dramatically decreased DN T cells in MRL/*lpr* spleens (Fig. 2C–D), which is consistent with the findings in peripheral blood (Supplemental Fig. 2). Despite the increased percentage of $CD4^+$ and $CD8^+$ T cells (Fig. 2C), the number of $CD4^+$ and $CD8^+$ T cells still slightly diminished (Fig. 2D) because the total number of T cells in the spleens of CTS-treated MRL/*lpr* mice was reduced for CTS administration (Fig. 2B). These results indicate that CTS treatment reduces DN T cell numbers specifically.

3.3. CTS treatment decreases DN T cell proliferation by suppressing STAT3 activation

STAT3, an important target of CTS [11,19], is necessary for T cell function [3] and contributes to SLE development [20,21]. Here, STAT3 activation was detected in T cells obtained from vehicle control or CTS-treated MRL/MpJ and MRL/*lpr* mice. The results showed that CTS impaired STAT3 phosphorylation in a dose-dependent manner (Fig. 3A) consistent with the reduced T cell accumulation. To confirm whether these effects were a direct consequence of CTS affecting T cell proliferation, T cells were isolated from spleen and cultured for 48 h in the presence of vehicle control or CTS with or without T cell-activating antibodies including anti-CD3 and anti-CD28 antibodies. The proliferation of both naïve and activated CTS-treated MRL/*lpr* T cell proliferation was blocked in the presence and absence of activating antibodies (Fig. 3B). In view of the dramatically reduced of DN T cell number for CTS treatment, we isolated the DN T cells from MRL/*lpr* spleens and found that CTS inhibited STAT3 activation dose-dependently (Fig. 3C) and the inhibitory effect was much better than that in all T cells (Fig. 3A). These results suggested that DN T cell subset is more sensitive to CTS treatment. Therefore, to further confirm the role of CTS in regulating DN T cell proliferation, we isolated and purified DN T cells from spleens of MRL/*lpr* mice. The DN T cells were cultured with or without CTS for 48 h. We found that the proliferation of DN T cell was inhibited for CTS treatment both in the presence and absence of anti-CD3 and anti-CD28 antibodies (Fig. 3D). Correspondently, the

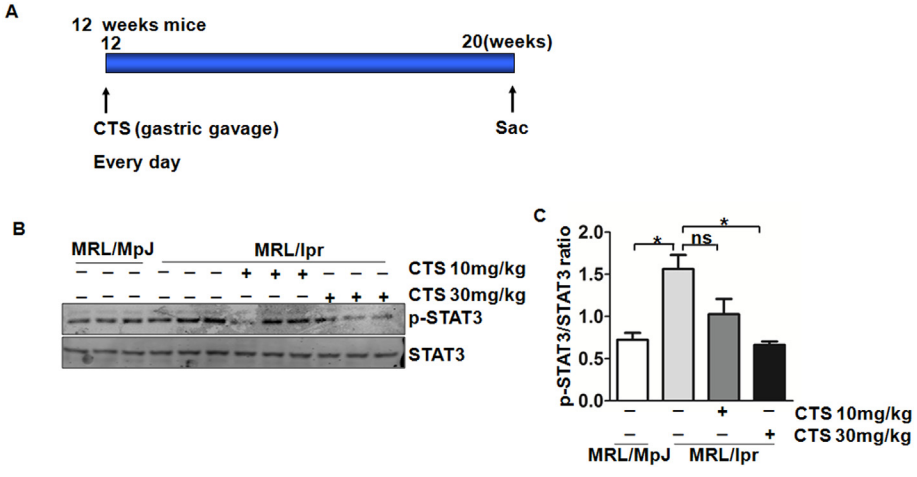

Fig. 1. CTS treatment reverses the increased STAT3 activation in lupus-prone MRL/*lpr* mice.
(A) MRL/*lpr* and MRL/MpJ female mice were randomly divided into three groups and administered with vehicle control, CTS 10 mg/kg or 30 mg/kg via gastric gavage every day for 9 weeks following the indicated scheme.
(B) Western blot assay indicating the phosphorylation of STAT3 (Y705) was suppressed dose-dependently in splenocyte isolated from CTS-treated MRL/*lpr* mice.
(C) Densitometry analysis of the indicated band intensities about STAT3 phosphorylation. n = 3, *p < 0.05.

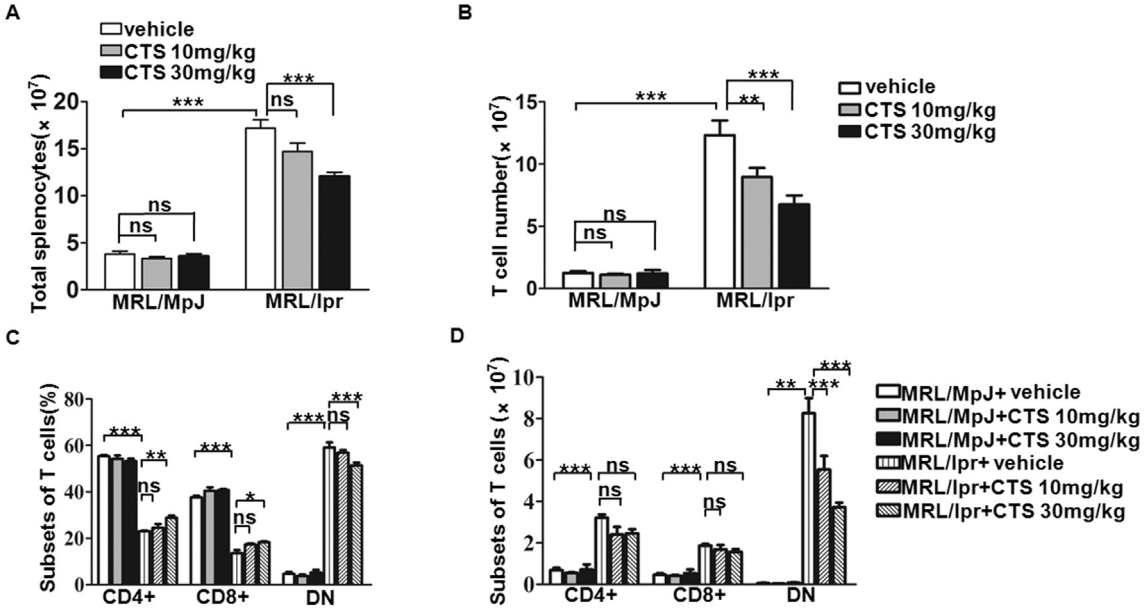

Fig. 2. CTS treatment reduces DN T cell number in MRL/*lpr* spleens.
(A) Total splenocyte counts from MRL/*lpr* and MRL/MpJ mice subjected to either vehicle control or CTS treatment (10 mg/kg and 30 mg/kg). n = 5, 7, or 9 mice/group. ***p < 0.001.
(B) Percentage of T cells from spleens of MRL/*lpr* and MRL/MpJ mice was analyzed via flow cytometry and the number was quantified according to the results of flow cytometry. n = 4, 5, 7, or 9 mice/group. **p < 0.01, ***p < 0.001.
(C–D) Flow cytometric analysis of $CD3^+$ gated cells to identify T cell subsets including $CD4^+$, CD8- and DN T cells from spleens of 20-week-old MRL/*lpr* and MRL/MpJ mice treated with vehicle control or CTS. The percentage and total number of T cell subsets were quantified according to the results of flow cytometry. n = 3, or 4 mice/group. *p < 0.05,**p < 0.01, ***p < 0.001.

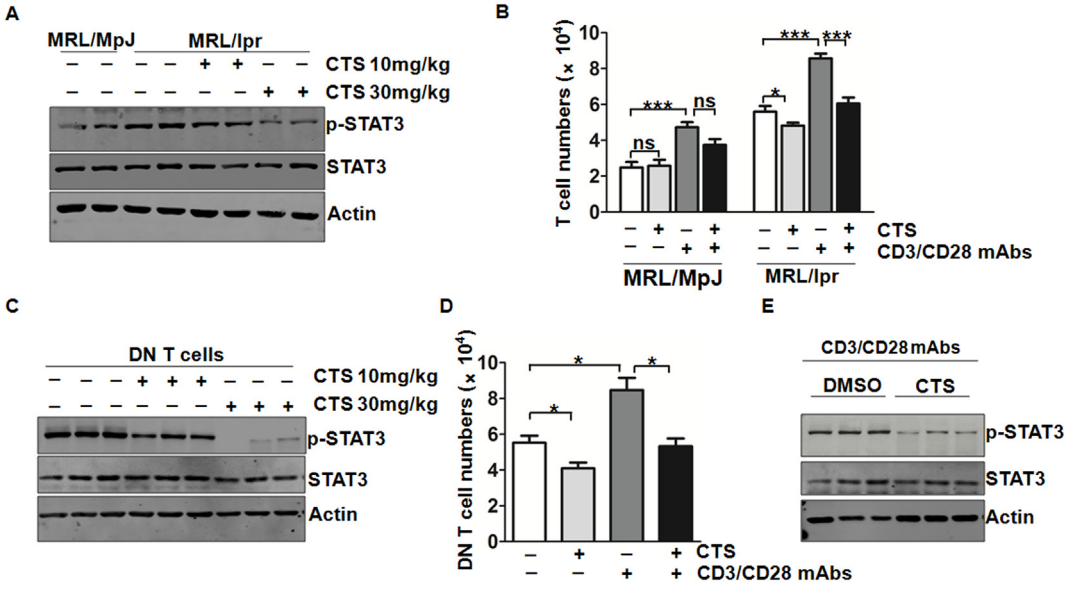

Fig. 3. CTS treatment blocks T cell proliferation by suppressing STAT3 activation.
(A) Western blot assay indicating the phosphorylation of STAT3 (Y705) was suppressed dose-dependently in total T cells isolated from CTS-treated MRL/*lpr* mice. Data were representative of three independent experiments.
(B) T cells obtained from spleens of MRL/*lpr* and MRL/MpJ mice were cultured (1×10^5 cells/well) in 96-well plates for 48 h in the presence of vehicle control or CTS (10 μM) with or without anti-CD3 and anti-CD28 antibodies to determine T cell proliferation. n = 3/group. *p < 0.05, ***p < 0.001.
(C) Western blot assay indicating the phosphorylation of STAT3 (Y705) was suppressed in DN T cells isolated from the spleens of CTS-treated MRL/*lpr* mice. Data were representative of three independent experiments.
(D) DN T cells isolated from spleens of MRL/*lpr* mice were cultured (1×10^5 cells/well) in 96-well plates for 48 h in the presence of vehicle control or CTS (10 μM) with or without anti-CD3 and anti-CD28 antibodies to determine T cell proliferation. n = 3/group. *p < 0.05.
(E) DN T cells isolated from spleens of MRL/*lpr* mice were cultured (1×10^5 cells/well) in 96-well plates for 48 h in the presence of vehicle control or CTS (10 μM) with anti-CD3 and anti-CD28 antibodies. Cells were then harvested and immunoblotted with anti-p-STAT3 and anti-STAT3 antibodies. Results showed that CTS inhibited STAT3 activation in DN T cells. Data were representative of three independent experiments.

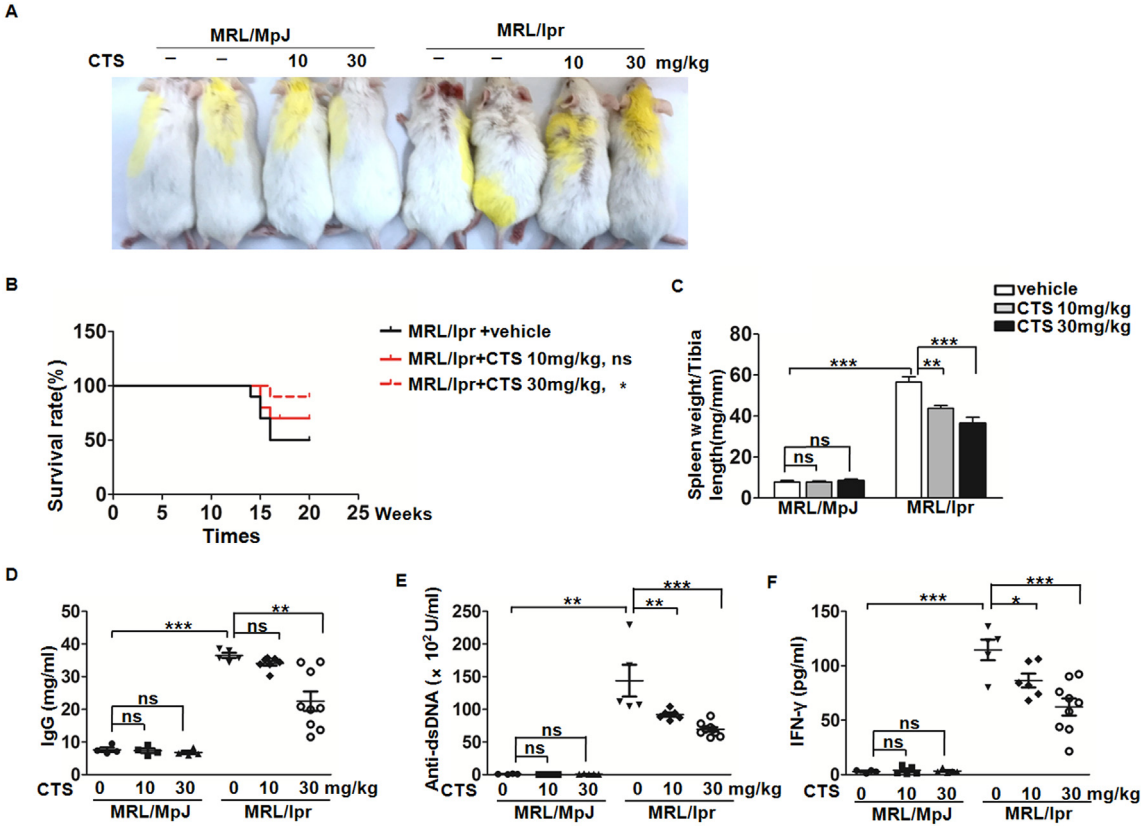

Fig. 4. CTS treatment significantly prevents SLE development in MRL/*lpr* mice.
(A) Representative photographs of skin lesions from MRL/*lpr* and MRL/MpJ mice treated with vehicle control or CTS for 9 weeks.
(B) Survival curve of 12- to 20-week-old MRL/*lpr* and MRL/MpJ mice subjected to either vehicle control or CTS treatment. n = 10 mice/group. *p < 0.05.
(C) Spleen weight to tibia length ratios from MRL/*lpr* and MRL/MpJ mice subjected to either vehicle control or CTS treatment (10 mg/kg and 30 mg/kg). n = 5, 7, or 9 mice/group. **p < 0.01, ***p < 0.001.
(D–F) Serum from 20-week-old MRL/*lpr* and MRL/MpJ mice treated with vehicle control or CTS was collected and assessed for the levels of total lgG (D), anti-dsDNA (E) and IFN-γ (F). n = 4–7, or 9 mice/group. *p < 0.05,**p < 0.01, ***p < 0.001.

phosphorylation of STAT3 was also been blocked in CTS-treated DN T cells (Fig. 3E). Taken together, these data suggest that CTS treatment inhibits DN T cell proliferation by suppressing STAT3 activation, which may contribute to the attenuated SLE progression.

3.4. CTS treatment significantly prevents spontaneous SLE development of MRL/lpr mice

Previous studies indicated that SLE patients exhibited increased number of DN T cells and which is corrected with SLE activity [22]. In SLE, pathogenic DN T cells showed defects in peripheral tolerance and contribute to the lymphadenopathy and systemic inflammation by inducing pro-inflammatory cytokines, activating other T cells and promoting the immunoglobulin production of B cells [23,24]. To better understand the contribution of impaired DN T cell proliferation for CTS treatment on the pathogenesis of lupus, we treated MRL/*lpr* and MRL/MpJ female mice with either vehicle control or CTS (10 mg/kg and 30 mg/kg) everyday beginning at 12 weeks of age. At 20 weeks, CTS-treated MRL/*lpr* mice exhibited dramatically alleviative skin lesions and showed an overall healthier appearance compared with vehicle control-treated MRL/*lpr* mice (Fig. 4A). In addition, the death of mice was recorded and survival curves showed an increased survival rate tendency in the 10 mg/kg CTS treatment group and a remarkable improvement in 30 mg/kg CTS treatment group compared to the vehicle control group up to 20 weeks (Fig. 4B). Moreover, in response to CTS, spleens isolated from MRL/*lpr* mice were substantially reduced in weight (Fig. 4C). We also measured the levels of total IgG and anti-dsDNA antibodies in serum, as they are the most essential autoantibodies in the pathogenesis of lupus [25] and found that CTS inhibited IgG (Fig. 4D) and anti-dsDNA (Fig. 4E) antibody accumulation in MRL/*lpr* mice. In addition, IFN-γ has been shown to be critical in lupus development, including local immune and inflammatory signaling processes [26,27]. Therefore, the IFN-γ level in serum was monitored and the results showed that CTS-treated MRL/*lpr* mice produced less IFN-γ (Fig. 4F) compared to untreated mice. Together, these data indicate that CTS is able to ameliorate lupus pathogenicity and prolongs the life of MRL/*lpr* mice by inhibiting DN T cell proliferation via regulating STAT3 activation.

3.5. CTS normalizes the renal structure and function of MRL/lpr mice

MRL/*lpr* mice develop immune complex glomerulonephritis that is similar to diffuse lupus nephritis in humans [28]. In this disease progresses, the kidney rapidly deteriorates. Therefore, renal structure and function were evaluated. Firstly, renal damage was assessed in PAS-stained sections using a composite score integrating immune cell infiltration, glomerular deposits, glomerular crescent formation, and endocapillary proliferation. The use of CTS significantly ameliorated the renal injury (Fig. 5A) and the histological damage index also indicated that kidney structure damage was relieved after CTS treatment (Fig. 5B). In addition, proteinuria is a major symptom of lupus nephritis [29]. To evaluate the effects of CTS on renal function, urine protein, albumin and creatinine levels were measured. As expected, we observed that CTS-administrated MRL/*lpr* mice exhibited a prominent reduction in total protein (Fig. 5C), as well as a reduction in ratio of albumin to creatinine (Fig. 5D) in urine, suggesting an overall

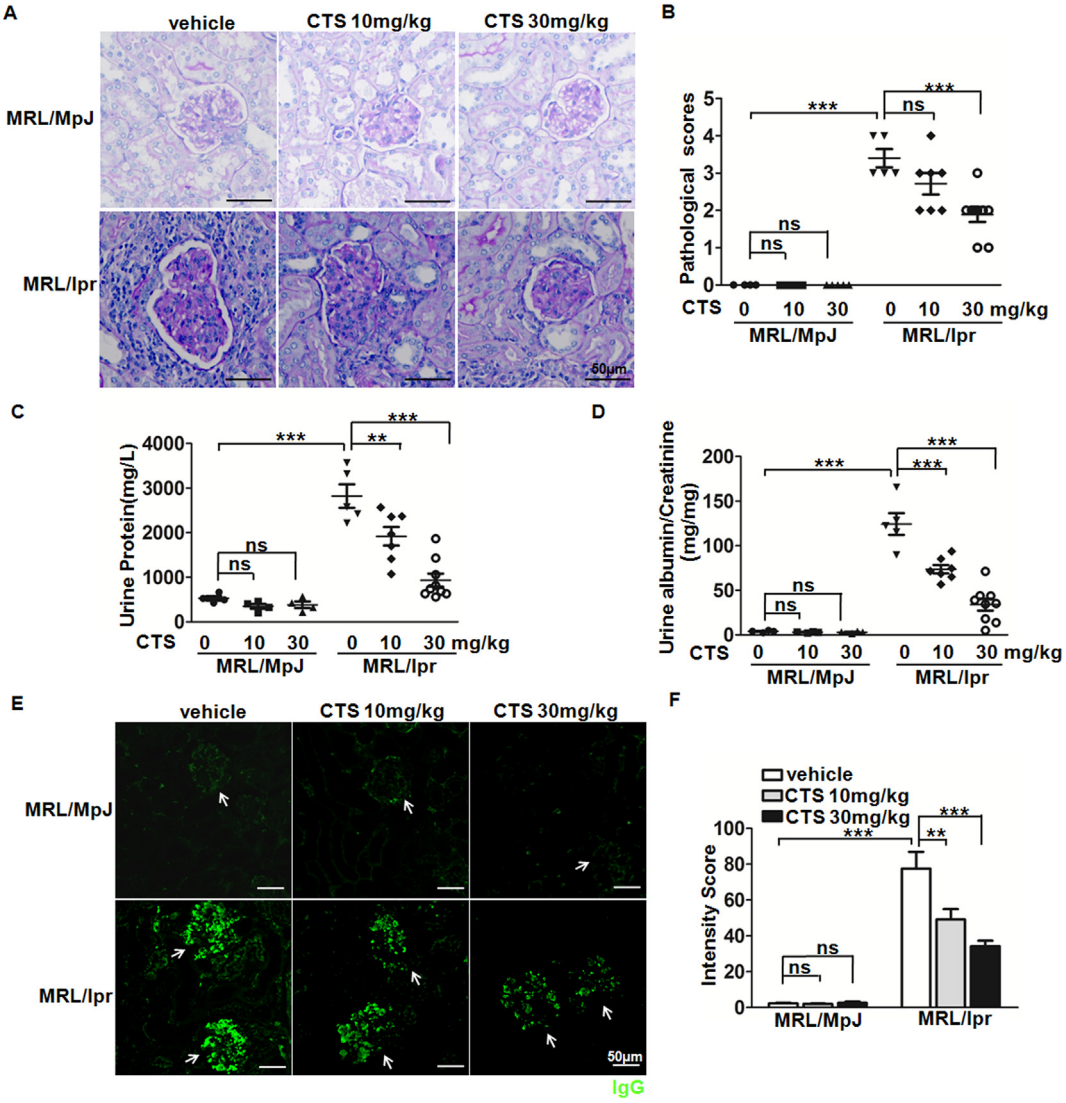

Fig. 5. CTS restores the renal structures and functions of MRL/*lpr* mice.
(A) Representative PAS-stained kidney sections and pathological scores (B) of 20-week-old MRL/*lpr* and MRL/MpJ mice treated with vehicle control or CTS. Scale bar = 50 μm, n = 4, 5, 7, or 9 mice/group. ***p < 0.001.
(C) CTS-treated MRL/*lpr* mice showing decreased urine protein excretion. n = 4, 5, 7, or 9 mice/group. **p < 0.01, ***p < 0.001.
(D) Albumin and creatinine in urine from MRL/*lpr* and MRL/MpJ mice treated with vehicle control or CTS were analyzed by ELISA and the ratio of albumin to creatinine was declined in response to CTS administration in MRL/*lpr* mice. n = 4, 5, 7, or 9 mice/group. ***p < 0.001.
(E) Immunofluorescence staining of IgG (green) deposition in the glomeruli of 20-week-old MRL/*lpr* and MRL/MpJ mice treated with vehicle control or CTS. Arrows indicate glomeruli. Representative images were shown. Scale bar = 50 μm, n = 4–7, or 9 mice/group.
(F) The mean fluorescence intensity of IgG accumulation was assessed by Image J. n = 4–7, or 9 mice/group. **p < 0.01, ***p < 0.001. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

improvement in renal function for CTS. Since the immune complex deposition in the kidney is an important pathological mechanism in lupus nephritis [3,30,31], we measured the IgG deposits in kidneys. As shown in Fig. 5E–F, IgG deposits in the glomerulus were notably decreased for CTS treatment in MRL/*lpr* mice. Collectively, these observations clearly define that CTS prevents and resolves renal damage in MRL/*lpr* mice.

3.6. CTS treatment attenuates renal inflammatory responses in MRL/lpr mice

Histologically, kidneys of MRL/*lpr* mice developed progressive crescent glomerulonephritis and substantial inflammatory cell infiltration. Consistent with CTS treatment protecting renal structure and functions, kidney inflammation assessed by H&E staining revealed that the use of CTS decreased the renal inflammatory response and the inhibitory effect of 30 mg/kg/d CTS was better than that of 10 mg/kg/d (Fig. 6A). Previous studies have shown that many cytokines are closely linked to the pathogenesis of SLE, including IFN family cytokines, IL-6, IL-17, CCL2 and so on [32–34]. For example, IFN-α, IFN-γ and CCL2 levels have been shown to be associated with activity of SLE [35] while IL-17 level reflects the inflammatory status of SLE patients independent of disease activity [36]. Therefore, the expression of pro-inflammatory cytokines or chemokines was analyzed via RT-PCR. As shown in Fig. 6B–G, the levels of IFN-α, IFN-β, IFN-γ, CCL2, IL-6 and IL-17A in kidneys were decreased in CTS-treated MRL/*lpr* mice relative to vehicle control-treated mice in a dose-dependent manner. Altogether, these results prove that CTS, an inhibitor of STAT3, inhibits lupus nephritis in MRL/*lpr* mice and it may be an attractive candidate drug for SLE.

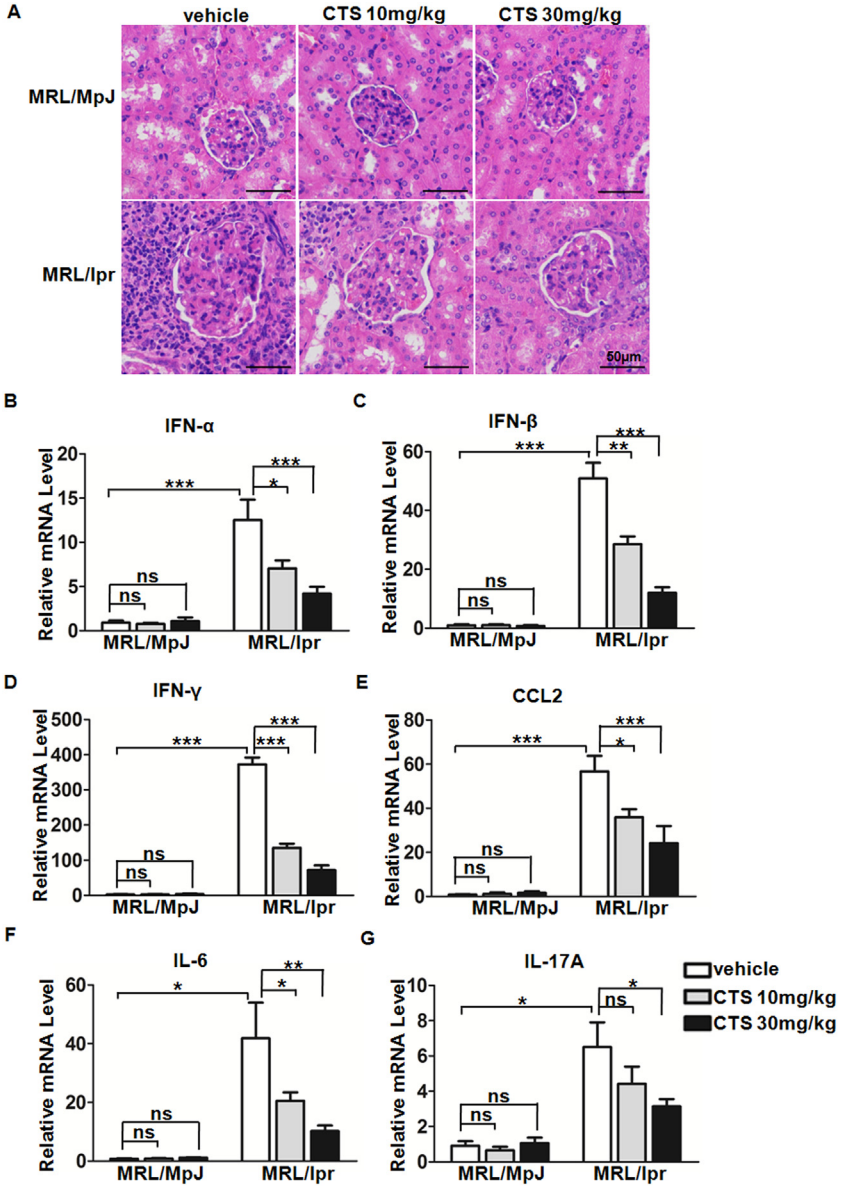

Fig. 6. Attenuated renal inflammatory responses after CTS treatment.
(A) Representative images of H&E staining from kidneys indicating reduced inflammatory cell infiltration after CTS treatment in MRL/*lpr* mice. Scale bar = 50 μm, n = 4–7, or 9 mice/group.
(B–G) The expression of pro-inflammatory cytokines and chemokines in kidneys was monitored via RT-PCR. n = 3 mice/group. *p < 0.05, **p < 0.01, ***p < 0.001.

4. Discussion

Small molecule inhibitors targeting signaling pathways responsible for the progression of SLE have played an important part in the development of therapeutic drugs for SLE [37]. Among the identified signaling pathways involved in SLE, JAK/STAT3 signaling is considered to be particularly important and previous studies have shown that impaired STAT3 signaling resulted in attenuated SLE development by regulating T or B cell function [3,9,37,38]. CTS, an effective inhibitor of STAT3, has been reported to possess significant antibacterial, anti-inflammatory as well as anti-cancer activities [39,40]. Recently, CTS has been demonstrated to have therapeutic effects for some chronic autoimmune diseases, such as rheumatoid arthritis (RA) [41,42], while its role in SLE development remains unknown.

T cells from patients with SLE display abnormal activation and contribute to the initiation and perpetuation of the autoimmune response, finally resulting in the development of organ damage. SLE T cells activate B cells, show inappropriate tissue homing and promote the secretion of inflammatory cytokines [43,44]. Evidence indicates that activation of T cells in SLE patients is accompanied with some increased signaling responses, such as PI3K/AKT and JAK/STAT3 pathways [44,45]. The JAK/STAT3 pathway is an important regulator of immune processes. Previous studies have shown that T cells isolated from SLE patients display elevated activation of STAT3, which plays a substantial role in the differentiation and migration of T cells, promoting B cell responses [46,47]. In our study, the enhanced activation of STAT3 in lupus T cells was normalized by CTS (Fig. 3A and C), which resulted in reduced T cell accumulation, especially DN T cells (Fig. 2B–D). Mechanistically, CTS inhibited DN T cell proliferation by suppressing STAT3 phosphorylation in vitro (Fig. 3D and E). On the basis, we found administration of CTS significantly inhibits SLE development in MRL/*lpr* mice in a dose-dependent manner with remissive skin lesions and splenomegaly, reduced autoantibody accumulation, as well as increased survival rate in CTS-treated MRL/*lpr* mice. In

addition, MRL/*lpr* mice displayed alleviative renal damage and inflammation, including decreased deposition of urine protein and immune complexes in the glomerulus as well as reduced infiltration of inflammatory cells and cytokines in the kidneys (Figs. 4–6). These data suggest that CTS treatment suppresses DN T cell proliferation by inhibiting STAT3 activation, resulting in ameliorated SLE symptoms.

Our data indicate that CTS targeting of downstream auto-reactive T cells may be sufficient to dampen SLE-associated organ injury. We found that auto-antibodies produced by B cells (IgG and anti-dsDNA) have also been suppressed by the use of CTS (Fig. 4D and E), which may be caused by decreased T cell accumulation resulting in interrupted B cell activation. A previous study has identified that STAT3 deficiency in B cells led to decreased B cell numbers and protected MRL/*lpr* mice from SLE [9]. However, here we found that the total B cell number exhibited a slight improvement in response to CTS administration, which suggested that CTS treatment and STAT3-specific deficiency in B cells had differences in the disparity of target cells and the inhibition range. In addition, subpopulations of B cells, including B1, B2 and Bregs, also play necessary roles in SLE [48,49] and there is a possibility that CTS influences the ratio of B cell subpopulations. In addition, we also can't deny that CTS may influence some other molecular targets to alleviate SLE. As a result, further studies are still needed to explore the other potential cell targets (such as B cells) and molecular targets of CTS in SLE treatment.

In conclusion, our findings highlight the role of CTS as a potential novel therapeutic drug for SLE. MRL/*lpr* mice treated with CTS showed ameliorated SLE progression, which is caused by the inhibition of STAT3 activation in DN T cells. Based on these findings, it may be possible to develop a promising therapeutic option for SLE.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.105677.

Abbreviations

CTS	cryptotanshinone
DN T cells	Double-negative T cells
H&E	hematoxylin and eosin
IF	immunofluorescence
PAS	periodic acid-schiff
SLE	systemic lupus erythematosus
STAT3	signal transducer and activator of transcription 3
WBC	white blood cells

Authors' contributions

Y.Z., Y.D., L.J. and C.P. designed the research, Y.Z., Y.D., L.J., Z.J. and Z.X. performed the experiments, collected and analyzed the data. C.P. and Y.Z. wrote the paper.

Declaration of Competing Interest

The authors have no conflicting financial interests.

Acknowledgements

We acknowledge pathologist Yueguang Du for performing the histological score of kidneys. We also thank American Journal Experts to polish the language.

Funding

This work was supported by the National Natural Science Foundation of China 81700022 to Y.Z., the National Key Research and Development Program of China 2018YFC1705501 and National Industry Public Welfare Science and Technology Project 201507001-4 to C.P., the National Natural Science Foundation of China 81873102 to Z.J. and the Research Fund of Zhejiang Chinese Medical University111100E013/001/001/029 and 111100E013/001/001/066 to Y.Z.

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