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Celastrol ameliorates lupus by promoting apoptosis of autoimmune T cells and preventing autoimmune response in MRL/lpr mice

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ABSTRACT

Objective Celastrol is a bioactive constituent extracted from *Tripterygium wilfordii* (thunder god vine). It has been demonstrated to have a therapeutic effect on experimental disease models for chronic inflammatory and immune disorders. In the present study, we investigated whether and how celastrol exerts a regulatory effect on the autoimmune response in MRL/lpr mice.

Methods We performed an in vivo study to determine the therapeutic effects of celastrol in MRL/lpr mice and then further investigated the underlying mechanism of celastrol in the regulation of the autoimmune response in MRL/lpr mice.

Results Celastrol showed a therapeutic effect in MRL/lpr mice by preventing the enlargement of the spleen and lymph nodes, alleviating renal injury, and reducing the levels of ANA and anti-double-stranded DNA antibodies. Furthermore, celastrol suppressed the in vivo inflammatory response in MRL/lpr mice by reducing the serum levels of multiple cytokines, including interleukin (IL)-6, tumour necrosis factor (TNF) and interferon (IFN)-γ, and the production of multiple antibody subsets, including total IgG, IgG_1 and IgG_{2b}. In vitro, celastrol reduced anti-CD3 antibody stimulation-induced T helper 1 and TNF-producing cells in CD4+ T cells of MRL/lpr mice. In addition, celastrol significantly affected B cell differentiation and prevented the generation of plasma cells from B cells in MRL/lpr mice by reducing the frequency of activated and germinal centre B cells. Celastrol treatment also affected T cell differentiation and significantly reduced central memory T cell frequencies in MRL/lpr mice. Importantly, celastrol treatment specifically promoted apoptosis of CD138+ but not CD138− T cells to suppress autoimmune T cell accumulation in MRL/lpr mice.

Conclusions Celastrol exerted therapeutic effects on lupus by specifically promoting apoptosis of autoimmune T cells and preventing the progression of autoimmune response.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ CD138+ T cells are the autoreactive T cells inducing autoimmune response that are accumulated in SLE murine model.
⇒ Although the role of glucocorticoids in SLE treatment is irreplaceable, substantial side effects of glucocorticoids in patients are also significant.

WHAT THIS STUDY ADDS

⇒ Celastrol had a therapeutic effect on MRL/lpr mice and specifically promoted CD138+ T cell apoptosis in MRL/lpr mice.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Celastrol may have an advantage over prednisone in suppressing autoimmune response and simultaneously not suppressing normal immune response.

INTRODUCTION

SLE is a chronic inflammatory autoimmune disease that is involved in multisystem injuries. It predominantly affects females, especially between puberty and menopause,[1 2] and is characterised by the production of autoantibodies,[3 4] including ANA and double-stranded DNA (dsDNA) antibodies. Epidemiological studies have demonstrated SLE to have a variable incidence rate and prevalence in different regions and environments worldwide.[5–7] Despite treatment advances in recent years, mortality in patients with SLE remains high, with significant geographical variations.[1 8 9] However, glucocorticoids are still used in clinical practice as a first-line treatment option and play an irreplaceable role in SLE treatment despite recent developments of therapeutic drugs such as belimumab and rituximab.[10–15] Glucocorticoids have shown significant therapeutic effects on SLE[10–13]; however, chronic usage of prednisone leads to irreversible damage or has substantial side effects in patients.[13 16–18] More treatment options with fewer side effects are required to substitute glucocorticoid treatment or reduce the dose of glucocorticoids, especially in patients who must be chronically administered glucocorticoids for SLE management.

Celastrol is a bioactive constituent extracted from *Tripterygium wilfordii* (thunder god vine). Celastrol has been demonstrated to have a therapeutic effect on experimental disease models for chronic inflammatory and immune disorders,[19–22] such as cancer and rheumatoid arthritis. Previous studies also have demonstrated celastrol could significantly alleviate lupus symptoms in SLE murine models.[22–24] However, the underlying mechanisms of the therapeutic effects of celastrol on lupus in SLE murine models have not been deciphered. Neutrophil extracellular traps (NETs) have been shown to play a key role in innate immunity to pathogens.[25] In addition, NETs have been reported to be involved in self-antigen exposure such as self-DNA from dying cells in autoimmune diseases.[25] Recent studies have indicated that celastrol could prevent in vitro NET formation induced by inflammatory stimuli by downregulating the SYK-MEK-ERK-NF-κB signalling pathway.[26] In the present study, we further investigated in detail the underlying mechanisms of celastrol for the treatment of SLE.

Autoantibodies initiate the autoimmune response and have a detrimental effect on multiple tissues and organs in patients with SLE.[27–29] It is believed that B cells play a central role in adaptive immune responses as they are specialised in producing antibodies. SLE is a disease that is highly variable and complex, and both T and B cells participate in the progress of SLE.[30–32] Recent studies have shown that T cells play a more important role in the progression of lupus in SLE murine models.[30 33 34] In the present study, we investigated whether celastrol could have a regulatory effect on the autoimmune response in Fas-deficient MRL/lpr mice and the mechanism of celastrol preventing autoimmune response.

MATERIALS AND METHODS

Mice

MRL/MPJ and MRL/lpr female mice were purchased from the Slac Laboratory (Shanghai, China). Mice were housed at 22±1°C with a relative humidity of 50%–60% and a 12-hour light/dark cycle.

Methods

Four-week-old female MRL/MPJ and MRL/lpr mice were acclimatised for 1 week. MRL/MPJ mice were used as controls (ddH_2O, n=9). MRL/lpr lupus mice were randomly grouped according to different treatments as follows: vehicle (ddH_2O, n=9), low dose of celastrol (CL, n=9) 2.5 mg/kg per day, high dose of celastrol (CH, n=9) 5.0 mg/kg per day and prednisone (PNS, n=6) 2.5 mg/kg per day. Celastrol (≥98%; high performance liquid chromatography (HPLC), batch no: DST200715-035; online supplemental figure 1A,B) was purchased from Desite Biotechnology (Chengdu, China). Oral administration by gavage was performed daily from 9 to 18 weeks of age. On the 19th or 20th week, mice were anaesthetised with 1% sodium pentobarbital (80 mg/kg) for serum collection and then euthanised by cervical dislocation with the following tissues harvested: lymph nodes and spleen (isolated and weighed) and kidneys (for histology).

Histology

To observe changes in renal pathology, paraformaldehyde-fixed kidneys were embedded in paraffin and then sectioned at 4 µm thickness. H&E, periodic acid Schiff and Masson trichrome staining were performed on the paraffin sections. Images of kidney tissues were obtained and analysed using Image-Pro Plus V.6.0 (Media Cybernetics, Rockville, Maryland, USA).

Measurement of total IgG, anti-dsDNA IgG and ANA in serum by ELISA

Total IgG, anti-dsDNA IgG and ANA levels in the serum of mice were measured using an ELISA kit (total IgG ELISA kit: Thermo Fisher; anti-dsDNA IgG and ANA ELISA kit: Alpha Diagnostic International, San Antonio, Texas, USA) according to the manufacturer's instructions. Optical density was determined at 450 nm absorbance using a microplate reader.

Measurement of multiple cytokines and antibody isotypes in the serum using the Luminex platform

Serum levels of multiple cytokines and antibody subtypes were measured using the Luminex assay kits (Thermo Fisher, USA). Measurements were performed according to the manufacturer's instructions and analysed on the Luminex platform.

Measurement of protein levels in the urine

Urine samples from individual mice were collected for 24 hours on the 16th week. Fresh urine samples were centrifuged at 300 *g* for 10 min and then stored at −80°C. Protein levels in urine samples were determined using the Coomassie brilliant blue dye-binding assay kit and performed according to the manufacturer's instructions (Biokits Tech, Beijing, China).

Cellular stimulation

Anti-CD3 antibody (5 µg/mL) or combined with 50 ng/mL PMA (phorbol 12-myristate 13-acetate), 1 µg/mL ionomycin and 2 µM monensin were used to stimulate and activate the cultured splenocytes from the mice.

Flow cytometry

Single-cell suspensions of splenocytes were obtained by filtering through a 70 µm cell strainer. Splenocytes were incubated on ice with CD16/CD32 monoclonal antibody (eBioscience, Thermo Fisher Scientific) for 15 min, and then red blood cells were lysed using lysis buffer (BD Biosciences). The following antibodies were used for staining of cellular surfaces for flow cytometry analysis according to the manufacturer's instructions: anti-CD3 PE-cy7, anti-CD3 APC-cy7, anti-CD4 FITC, anti-CD8 PerCP, anti-CD19 APC-cy7, anti-CD19 PE-cy7, anti-CD138 PE, anti-CD69 APC, anti-CD25 APC, anti-CD44 PE, anti-CD62L APC, anti-B220 PerCP, anti-GL-7 APC, anti-major histocompatibility complex (MHC)-II FITC, anti-CD23

APC, anti-CD21 PE and anti-CD69 PE. Vendor and lot numbers are provided in online supplemental table 1 of this manuscript. Splenocytes were fixed and permeabilised (Fixation/Permeabilization Solution, eBioscience, Thermo Fisher Scientific) before intracellular staining. Cells were intracellularly stained with the following antibodies according to the manufacturer's instructions: anti-Foxp3 PE, anti-interleukin (IL)-4 APC, anti-interferon (IFN)-γ PE-cy7 and anti-tumour necrosis factor (TNF) PE. Annexin V-FITC and 7-AAD (7-aminoactinomycin D) PerCP were used to determine the live and apoptotic cell populations. The details of all the antibodies used for flow cytometry are shown in online supplemental table 1. Flow cytometry data were analysed using FlowJo V.10.6 software (Tree Star).

Statistical analysis

Data from all experiments were presented as mean±SD and analysed using the SPSS 17.0 software. Comparisons between the groups were performed for statistical significance using the Kruskal-Wallis H test followed by Dunn's post-test for analysis of non-parametric data and one-way analysis of variance followed by Tukey's post-hoc test for analysis of parametric data. Mann-Whitney U test was used to assess statistical differences between two groups. Differences with p values less than 0.05 were considered statistically significant.

RESULTS

Celastrol ameliorates lupus symptoms in MRL/lpr mice

Compared with MRL/MPJ mice, significant enlargement of the spleen and lymph nodes, increased levels of ANA and anti-dsDNA IgG antibodies in the serum, obvious renal injuries, and elevated protein levels in the urine were observed in vehicle-treated MRL/lpr mice (figure 1). After administration of celastrol (2.5 mg/kg CL and 5.0 mg/kg CH), increased weight of the spleen and lymph nodes, elevated levels of ANA and anti-dsDNA IgG antibodies in the serum, and elevated protein levels in the urine of MRL/lpr mice were significantly ameliorated (figure 1A, D–E). Additionally, obvious renal injuries in vehicle-treated MRL/lpr mice, such as hyaline deposits, interstitial and perivascular cellular inflammatory infiltration, cellular crescent formation, glomerular fibrosis, glomerulosclerosis, and tubular cell necrosis, were also significantly alleviated (figure 1B,C) in MRL/lpr mice after celastrol treatment (2.5 mg/kg CL and 5.0 mg/kg CH). These results demonstrated that celastrol could have a significant therapeutic effect on lupus in MRL/lpr mice.

Celastrol reduces antibody secretion

In vivo inflammation induced by immune complexes and activated complement results in multiple organ injuries to promote SLE development.[35] We observed multiple antibody subsets in the serum, that is, total IgG, IgG_1, IgG_{2a}, IgG_{2b}, IgG_3, IgM, IgA and IgE levels were increased in MRL/lpr lupus mice compared with MRL/MPJ mice (figure 2). After celastrol administration (5.0 mg/kg CH), total IgG, IgG_1 and IgG_{2b} levels in the serum of MRL/lpr mice were significantly reduced compared with vehicle-treated MRL/lpr mice (figure 2A–B,D).

Celastrol significantly affects B cell differentiation

Compared with MRL/MPJ mice, B cells in the splenocytes of vehicle-treated MRL/lpr mice were significantly reduced (figure 3A). Additionally, we observed the frequency of follicular B cells was significantly reduced (figure 3B); however, the frequency of marginal B cells was increased in vehicle-treated MRL/lpr mice compared with MRL/MPJ mice (figure 3B). Both MHC-II expression levels in B cells and the frequency of activated B cells in vehicle-treated MRL/lpr mice were significantly increased compared with MRL/MPJ mice (figure 3C,D); however, no significant differences in the frequency of germinal centre (GC) B cells between vehicle-treated MRL/lpr mice and MRL/MPJ mice (figure 3E) were observed. Importantly, the frequency of plasma cells in CD3– cells of MRL/lpr mice was much higher compared with MRL/MPJ mice (figure 3F).

Celastrol administration (5.0 mg/kg CH) significantly increased B cell frequency in the splenocytes of MRL/lpr mice (figure 3A) and markedly reduced the frequency of activated B cells in total B cells of MRL/lpr mice (figure 3D). Furthermore, GC B cell frequency was significantly reduced in celastrol-treated MRL/lpr mice (5.0 mg/kg CH) compared with vehicle-treated MRL/lpr mice (figure 3E). Plasma cell frequencies in CD3– cells of MRL/lpr mice after oral administration of celastrol (2.5 mg/kg CL and 5.0 mg/kg CH) were found to be dramatically reduced (figure 3F); however, celastrol administration did not show significant effects on the frequencies of both follicular and marginal B cells in B cells of MRL/lpr mice (figure 3B). Celastrol treatment also failed to significantly reduce MHC-II expression in B cells of MRL/lpr mice (figure 3C).

Celastrol suppresses the inflammatory response

Multiple cytokine levels, including IFN-γ, IL-6, IL-12 and TNF, in the serum were significantly increased in MRL/lpr mice compared with MRL/MPJ mice (figure 4A). In addition, the T helper (Th) 1 cell frequency of CD4+ T cells in vehicle-treated MRL/lpr mice was significantly increased after 5-hour in vitro stimulation of splenocytes with anti-CD3 antibody compared with MRL/MPJ mice (figure 4B,C). However, the Th2 cell frequency of CD4+ T cells was reduced in vehicle-treated MRL/lpr mice compared with MRL/MPJ mice (figure 4B,C). Accordingly, the ratio of Th1 to Th2 in vehicle-treated MRL/lpr mice was significantly increased compared with MRL/MPJ mice (figure 4B,C). Interestingly, the number of TNF-producing cells in CD4+ T cells of MRL/lpr mice was markedly reduced compared with MRL/MPJ mice after 5-hour in vitro stimulation of splenocytes with anti-CD3 antibody despite the higher TNF serum levels in vivo in MRL/lpr mice (figure 4D). Additionally, regulatory

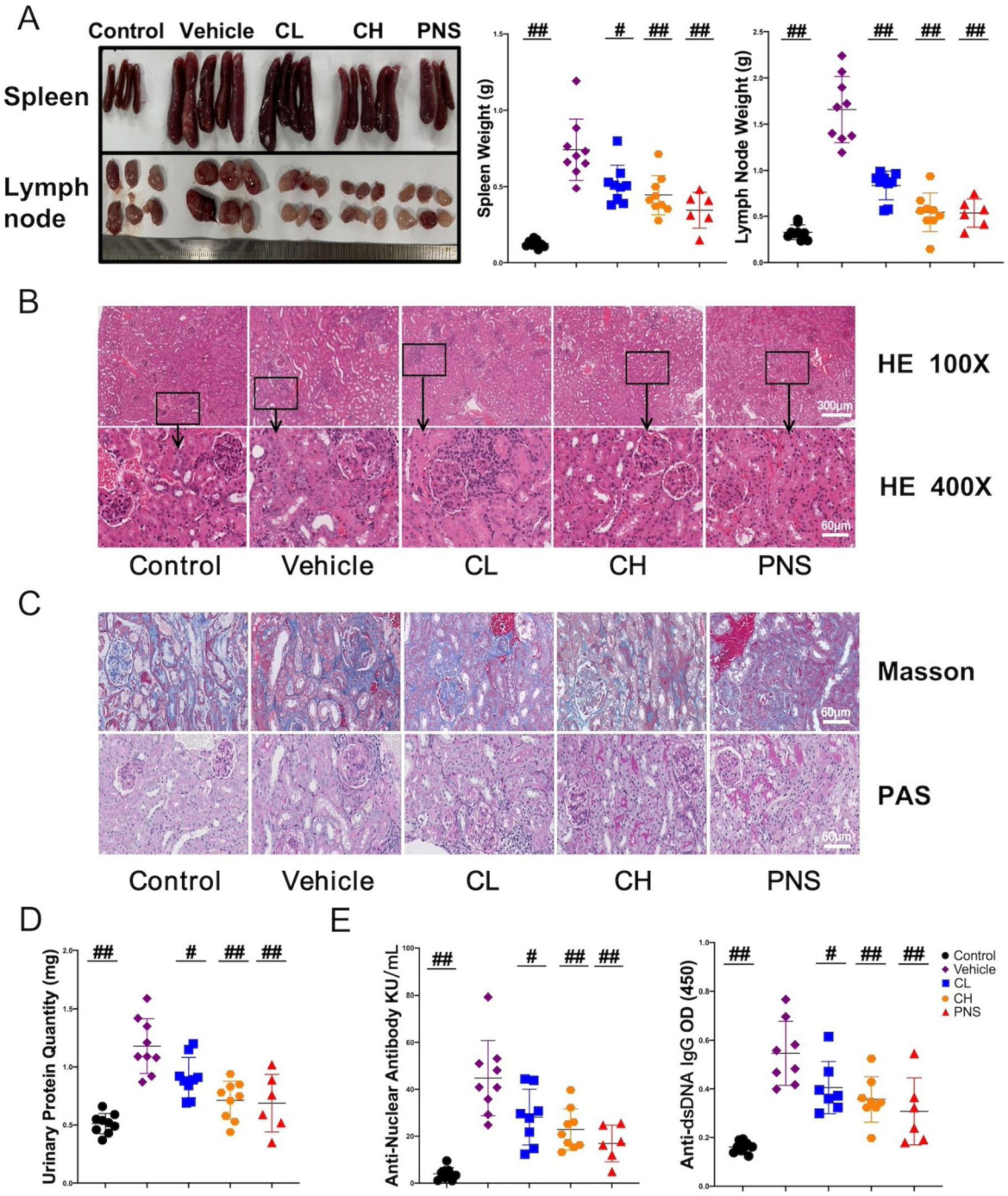

Figure 1 (A) Image of the spleen (top panel) and lymph nodes (bottom panel), with scatter plots showing the weight of the spleen and lymph nodes. (B) Renal tissue pathological sections were stained with HE, original magnification: 100× (top panel) and 400× (bottom panel); scale bar: 300 µm and 60 µm. (C) Renal tissue pathological sections were stained with PAS and Masson, original magnification: 400×; scale bar: 60 µm. (D) Scatter plots denote urinary protein quantity. (E) Scatter plots denote ANA and anti-dsDNA antibody levels in the serum of mice. Data presented as mean±SD (n=6–9 mice per group) from two to three independent experiments: #p<0.05, ##p<0.01 by one-way analysis of variance. CH, high-dose celastrol; CL, low-dose celastrol; dsDNA, double-stranded DNA; OD, optical density; PAS, periodic acid Schiff; PNS, prednisone.

T (Treg) cell frequency in CD4+ T cells was reduced in vehicle-treated MRL/lpr mice (figure 4E).

In vivo inflammation of MRL/lpr mice was significantly reduced by celastrol administration. After oral administration of 2.5 mg/kg (CL) celastrol, serum levels of TNF in MRL/lpr mice were significantly reduced compared with vehicle-treated MRL/lpr mice (figure 4A). IFN-γ and IL-6 levels in the serum of MRL/lpr mice treated with 5.0 mg/kg (CH) of celastrol showed an even more striking decrease (figure 4A). Furthermore, Th1 cell frequencies in CD4+ T cells of celastrol-treated MRL/lpr mice

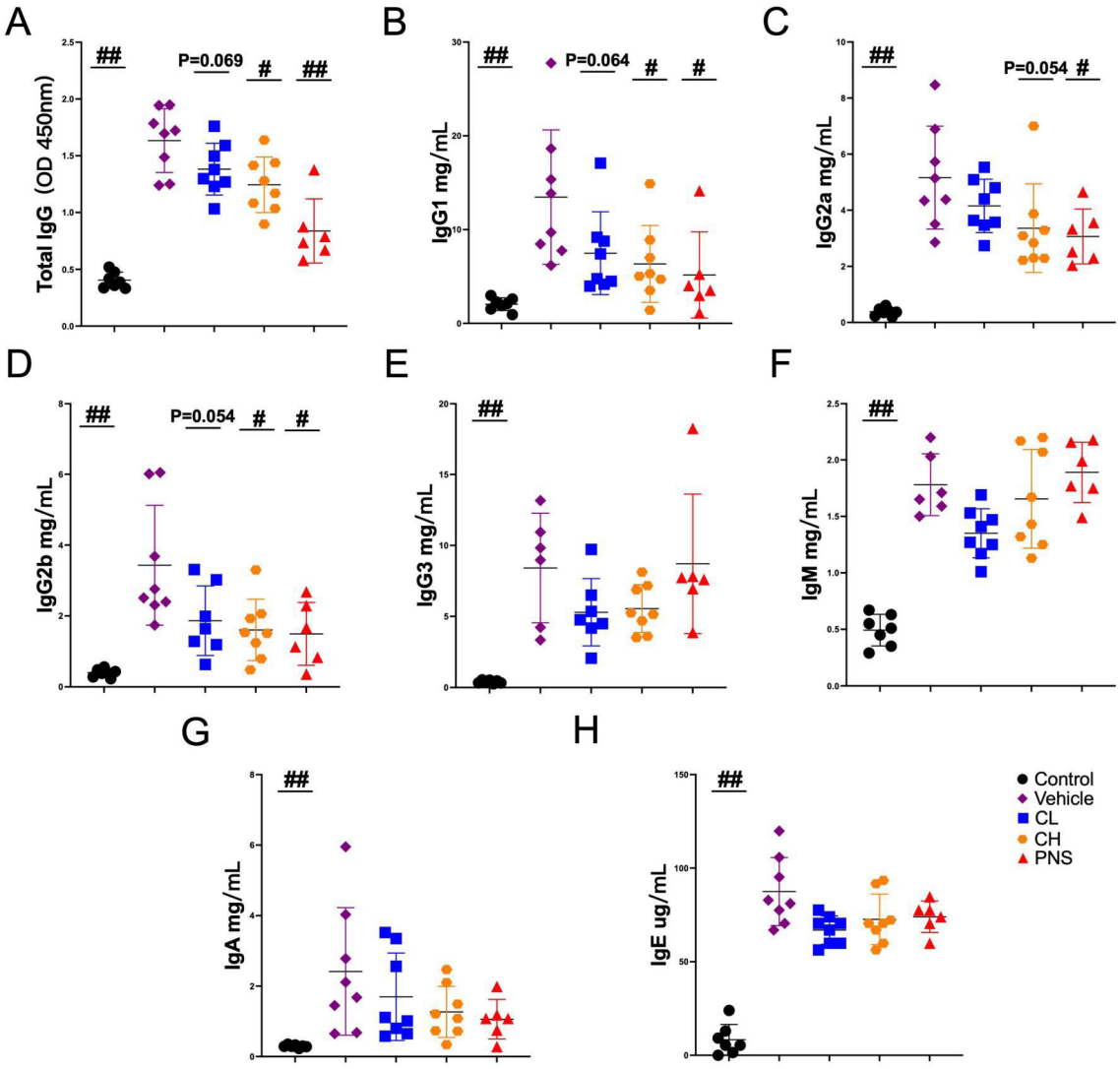

Figure 2 Scatter plots denote (A) total IgG, (B) IgG_1, (C) IgG_{2a}, (D) IgG_{2b}, (E) IgG_3, (F) IgM, (G) IgA and (H) IgE levels in the serum of mice. Data presented as mean±SD (n=6–8 mice per group) from two to three independent experiments: #p<0.05, ##p<0.01 by Kruskal-Wallis H test (D) or one-way analysis of variance. CH, high-dose celastrol; CL, low-dose celastrol; OD, optical density; PNS, prednisone.

(2.5 mg/kg CL and 5.0 mg/kg CH) were also significantly reduced after 5-hour in vitro stimulation of splenocytes with anti-CD3 antibody compared with vehicle-treated mice (figure 4B,C). Likewise, Th2 cell frequency in CD4+ T cells (5.0 mg/kg CH) was also simultaneously reduced in splenocytes of celastrol-treated MRL/lpr mice (figure 4B,C) after 5-hour in vitro anti-CD3 antibody stimulation. However, the ratio of Th1 to Th2 after anti-CD3 antibody stimulation in vitro was not significantly affected by celastrol administration (figure 4C). In addition, the frequency of TNF-producing cells in CD4+ T cells of celastrol-treated MRL/lpr mice (2.5 mg/kg CL and 5.0 mg/kg CH) was also strikingly reduced compared with vehicle-treated mice (figure 4D). However, the frequency of Treg cells in CD4+ T cells of MRL/lpr mice was not significantly affected by celastrol administration (figure 4E).

Celastrol treatment reduces the frequency of Tcm in T cells

Compared with MRL/MPJ mice, CD3+ T cell frequency was significantly increased in the splenocytes of vehicle-treated MRL/lpr mice (figure 5A). Furthermore, double-negative (DN) T cells were also increased in CD3+ T cells of MRL/lpr mice (figure 5B). However, CD4+ and CD8+ T cell frequencies in the splenocytes of vehicle-treated MRL/lpr mice were reduced compared with MRL/MPJ mice (figure 5B). Additionally, CD69+ cells, central memory (Tcm) and effector memory (Tem) T cell frequencies in CD3+ T cells of vehicle-treated MRL/lpr mice were significantly increased compared with MRL/MPJ mice (figure 5C,D). However, CD44–CD62L– and naïve T (Tn) cell frequencies in CD3+ T cells of vehicle-treated MRL/lpr mice were reduced (figure 5D).

Oral administration of celastrol did not significantly prevent the accumulation of T cells in MRL/lpr mice (figure 5A). However, DN T cell accumulation in

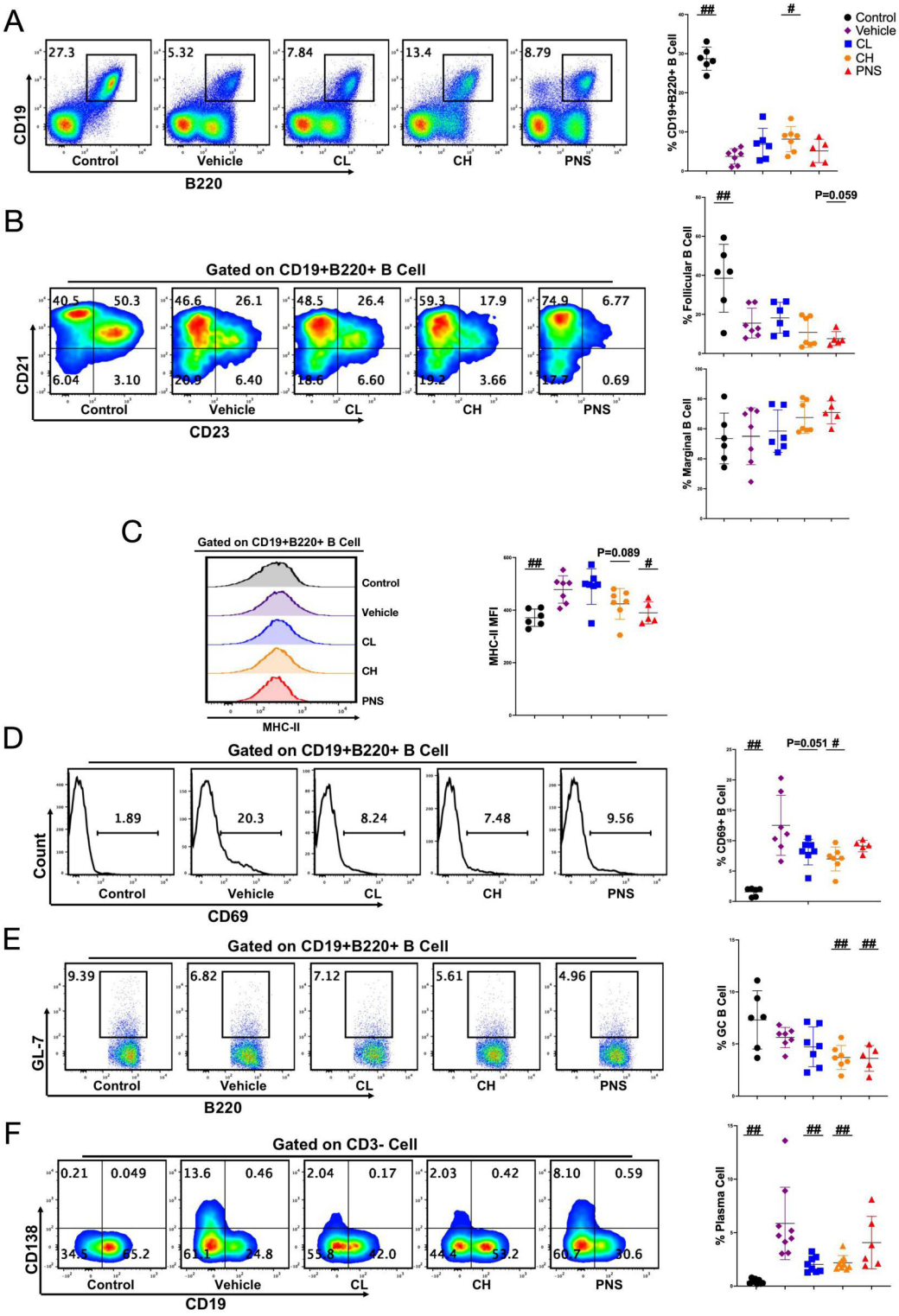

Figure 3 (A) Representative flow cytometry analyses and scatter plots denoting the frequencies of CD19+B220+ B cells in the splenocytes of mice. (B) Representative flow cytometry analyses and scatter plots denoting the frequencies of follicular (gated on CD19+B220+CD23+CD21int) and marginal zone (gated on CD19+B220+CD21+CD23–) B cells in B cells of mice. (C) Representative flow cytometry analyses and scatter plots denoting MHC-II MFI for MHC-II expression levels in B cells (gated on CD19+B220+) of mice. (D) Representative flow cytometry analyses and scatter plots denoting the frequencies of CD69+ cells in B cells (gated on CD19+B220+) of mice. (E) Representative flow cytometry analyses and scatter plots denoting the frequencies of GC B cells (gated on CD19+B220+GL-7+) in B cells of mice. (F) Representative flow cytometry analyses and scatter plots denoting the frequencies of plasma cells (gated on CD138+CD19–) in CD3– cells of mice. Data presented as mean±SD (n=5–9 mice per group) from two to three independent experiments: #p<0.05, ##p<0.01 by Kruskal-Wallis H test (F) or one-way analysis of variance. CH, high-dose celastrol; CL, low-dose celastrol; GC, germinal centre; MFI, mean fluorescence intensity; MHC, major histocompatibility complex; PNS, prednisone.

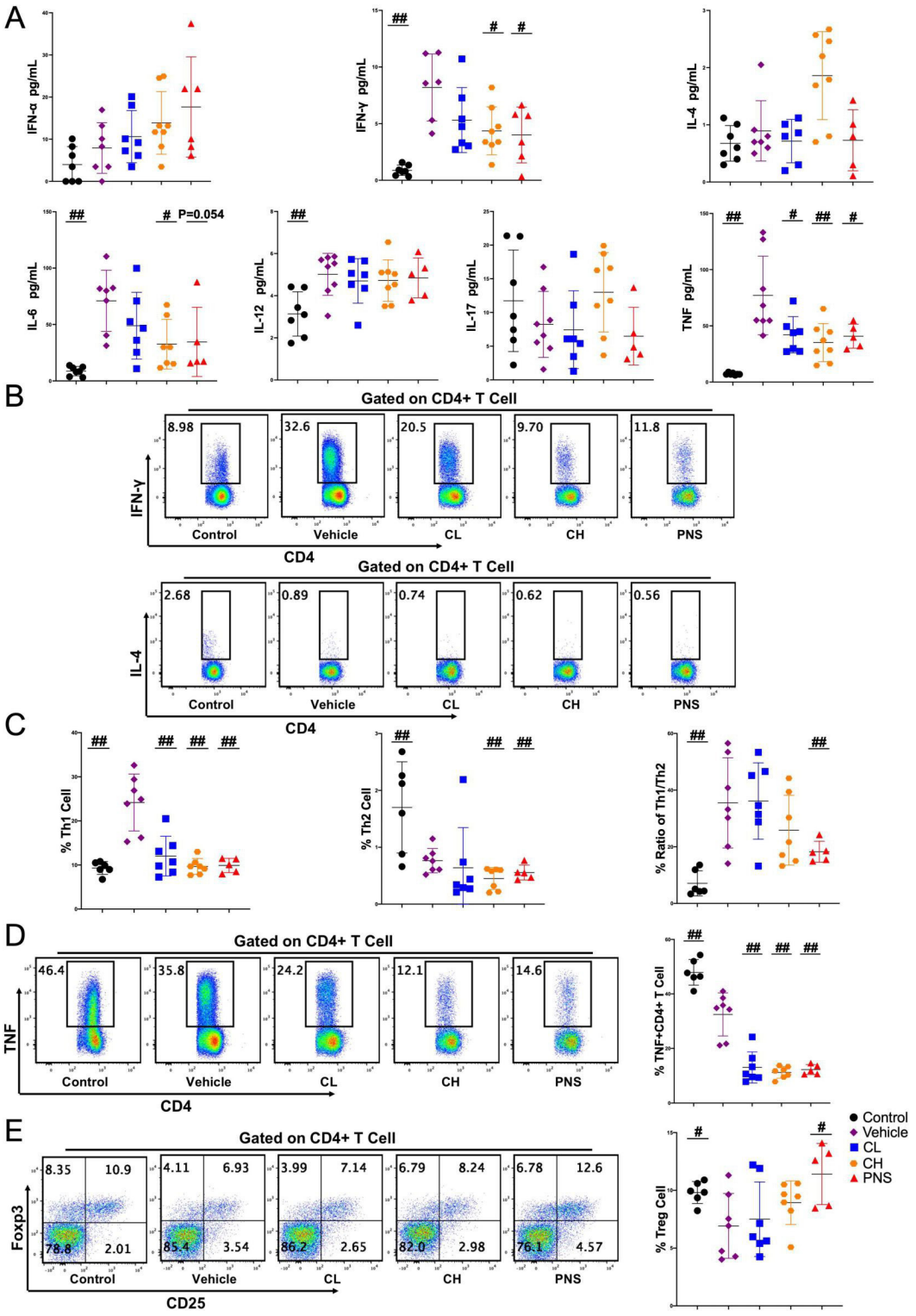

Figure 4 (A) Scatter plots denoting the levels of multiple cytokines in the serum of mice. (B) Representative flow cytometry analyses denoting the frequencies of Th1 (gated on IFN-γ+CD4+) and Th2 (gated on IL-4+CD4+) cells in CD4+ T cells of anti-CD3 antibody-stimulated splenocytes of MRL/lpr mice (in vitro for 5 hours). (C) Scatter plots denoting the Th1 and Th2 cell frequencies in CD4+ T cells of anti-CD3 antibody-stimulated splenocytes of MRL/lpr mice (in vitro for 5 hours) and the ratio of Th1 to Th2 cells. (D) Representative flow cytometry analyses and scatter plots denoting the frequencies of TNF-producing cells in CD4+ T cells of anti-CD3 antibody-stimulated splenocytes of MRL/lpr mice (in vitro for 5 hours). (E) Representative flow cytometry analyses and scatter plots denoting the frequencies of Treg cells (Foxp3+CD25+CD4+) in CD4+ T cells of mice. Data presented as mean±SD (n=5–9 mice per group) from two to three independent experiments: #p<0.05, ##p<0.01 by Mann-Whitney U test (comparison of Th2 frequency between vehicle and CL in (C) or one-way analysis of variance. CH, high-dose celastrol; CL, low-dose celastrol; IFN, interferon; IL, interleukin; PNS, prednisone; Th1, T helper 1; Th2, T helper 2; TNF, tumour necrosis factor; Treg, regulatory T cells.

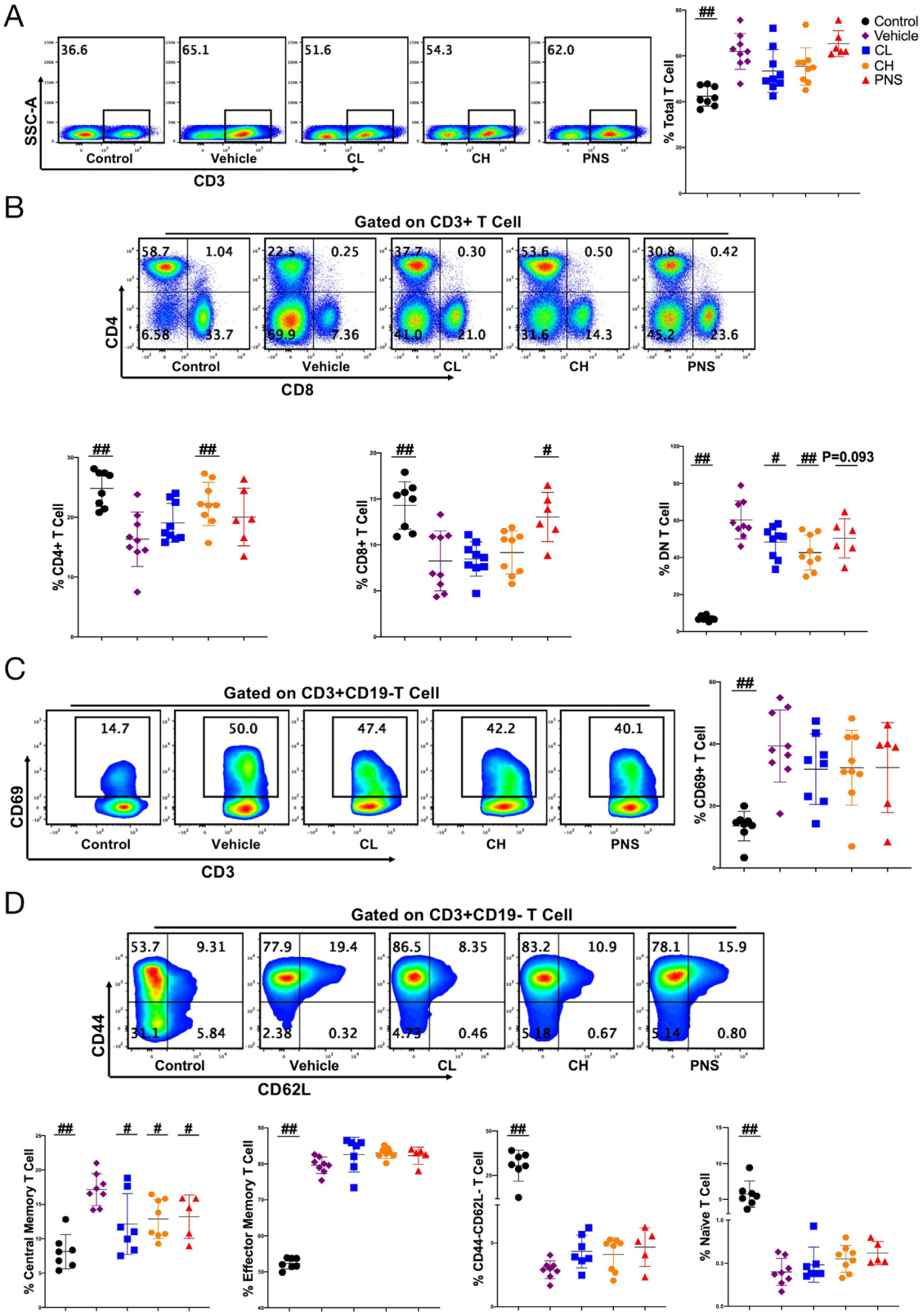

Figure 5 (A) Representative flow cytometry analyses and scatter plots denoting the frequencies of CD3+ total T cells in the splenocytes of mice. (B) Representative flow cytometry analyses and scatter plots denoting the frequencies of CD4+ and CD8+ T cells in the splenocytes of mice and the frequencies of DN T cells among CD3+ T cells. (C) Representative flow cytometry analyses and scatter plots denoting the frequencies of CD69+ cells in CD3+ T cells of mice. (D) Representative flow cytometry analyses and scatter plots denoting the frequencies of central memory (CD44+CD62L+), effector memory (CD44+CD62L−), naïve (CD44−CD62L+) and CD44−CD62L− T cells in CD3+ T cells of mice. Data presented as mean±SD (n=6–9 mice per group) from two to three independent experiments: #p<0.05, ##p<0.01 by one-way analysis of variance. CH, high-dose celastrol; CL, low-dose celastrol; DN, double-negative; PNS, prednisone; SSC-A, side scatter-area.

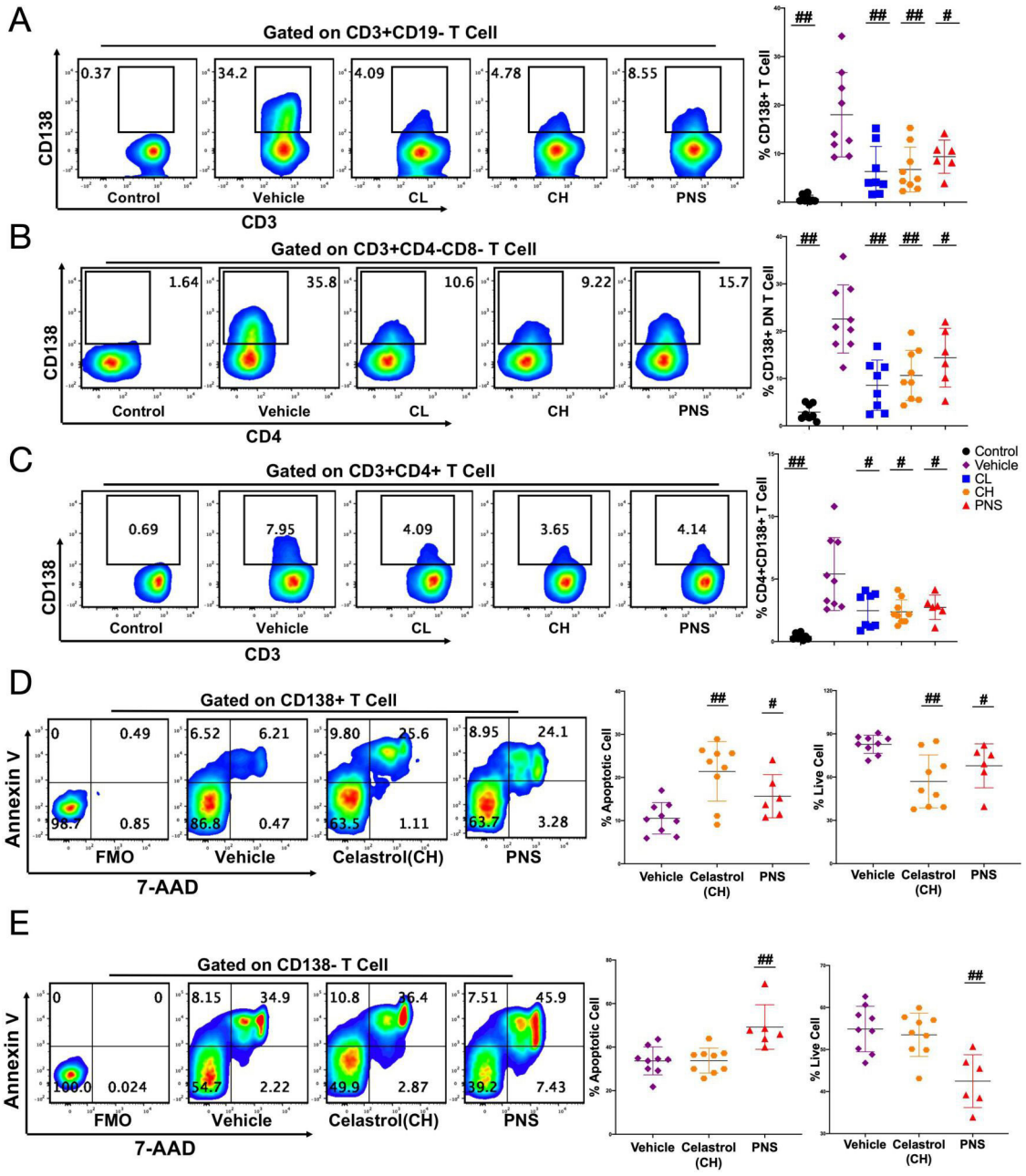

Figure 6 (A) Representative flow cytometry analyses and scatter plots denoting the frequencies of CD138+ cells among CD3+ T cells in mice. (B) Representative flow cytometry analyses and scatter plots denoting the frequencies of CD138+ cells in DN T cells of mice. (C) Representative flow cytometry analyses and scatter plots denoting the frequencies of CD138+ cells in CD4+ T cells of mice. (D) Representative flow cytometry analyses and scatter plots denoting the frequencies of apoptotic and live cells in CD138+ T cells of mice. (E) Representative flow cytometry analyses and scatter plots denoting the frequencies of apoptotic and live cells in CD138– T cells of mice. Data presented as mean±SD (n=6–9 mice per group) from two to three independent experiments: #p<0.05, ##p<0.01 by Kruskal-Wallis H test (E) or one-way analysis of variance. 7-AAD, 7-aminoactinomycin D; CH, high-dose celastrol; CL, low-dose celastrol; DN, double-negative; FMO, fluorescence minus one; PNS, prednisone.

CD3+ T cells of MRL/lpr mice after celastrol treatment (2.5 mg/kg CL and 5.0 mg/kg CH) was significantly alleviated compared with vehicle-treated MRL/lpr mice (figure 5B). In addition, CD4+ T cell frequency in the splenocytes showed an obvious increase in MRL/lpr mice after celastrol treatment (5.0 mg/kg CH) compared with vehicle-treated MRL/lpr mice (figure 6B). Celastrol treatment, however, did not show a significant effect on the frequency of activated T cells in MRL/lpr mice (figure 5C). Tcm cell frequencies in MRL/lpr mice after celastrol treatment (2.5 mg/kg CL and 5.0 mg/kg CH) were significantly reduced compared with vehicle-treated MRL/lpr mice, but Tem, Tn and CD44–CD62L– T cell frequencies in CD3+ T cells of MRL/lpr mice were not significantly affected by celastrol treatment (figure 5D).

Celastrol specifically promotes apoptosis of CD138+ T cells

Fas deficiency and subsequent reduced apoptosis in CD138+ T cells result in CD138+ T cell accumulation in MRL/lpr mice.[34] We observed CD138+ T cell accumulation in CD3+ T cells of vehicle-treated MRL/lpr mice but not in MRL/MPJ mice (figure 6A). CD138+ cell frequencies were also significantly increased in CD4+ and DN T cells of vehicle-treated MRL/lpr mice compared with MRL/MPJ mice (figure 6B,C). However, CD138+ cell frequencies in CD3+ T cells and T cell subsets (DN and CD4+ T cells) were significantly reduced in MRL/lpr mice after treatment of celastrol (2.5 mg/kg CL and 5.0 mg/kg CH) compared with vehicle-treated MRL/lpr mice (figure 6A–C). In addition, we observed the frequency of apoptotic cells was increased but the frequency of live cells was reduced in CD138+ T cells of celastrol-treated (5.0 mg/kg CH) MRL/lpr mice compared with vehicle-treated MRL/lpr mice (figure 6D). However, the frequency of apoptotic cells was not increased and the frequency of live cells was also not reduced in CD138− T cells of celastrol-treated (5.0 mg/kg CH) MRL/lpr mice compared with vehicle-treated MRL/lpr mice (figure 6E).

DISCUSSION

In the present study, celastrol administration had a therapeutic effect on MRL/lpr mice by significantly preventing the enlargement of the spleen and lymph nodes, in addition to alleviating renal injuries and reducing the production of ANA and anti-dsDNA antibodies. Celastrol treatment also showed significant effects on suppressing in vivo inflammation in MRL/lpr mice by reducing serum levels of multiple cytokines, including IL-6, TNF and IFN-γ, and multiple antibody subsets, including total IgG, IgG_1 and IgG_{2b}, in MRL/lpr mice. Celastrol treatment also reduced Th1 and TNF-producing cell frequencies in CD4+ T cells of MRL/lpr mice to suppress inflammation.

Celastrol further inhibited the accumulation of DN and CD138+ T cells in MRL/lpr mice and significantly decreased Tcm frequency in CD3+ T cells of MRL/lpr mice. Importantly, celastrol specifically promoted CD138+ T cell apoptosis by increasing apoptosis levels of CD138+ T cells but not CD138− T cells in MRL/lpr mice. In addition, celastrol prevented plasma cell formation from B cells in MRL/lpr mice by reducing the frequency of activated and germinal B cells in the B cell population. Prednisone also exerted a strikingly therapeutic effect on MRL/lpr mice in our present study. However, in contrast to celastrol-specific effect on CD138+ T cells, prednisone had an effect on both CD138+ and CD138− T cells and promoted both CD138+ and CD138− T cell apoptosis by increasing apoptosis levels of CD138+ and CD138− T cells.

Syndecan-1/CD138 is a marker of plasma cells in lymphocytes that are believed to originate from B cells.[36 37] CD138+ T cells, which express both CD3 and CD138, have been reported recently to be plasmablastic B cell neoplasms as observed in clinical cases.[38] However, these abnormal CD138+ cells have also been observed in murine SLE models.[34 39 40] Previous studies have demonstrated CD138+ T cells in MRL/lpr mice to significantly promote autoantibody production both in vivo and in vitro, indicating CD138+ T cells are the autoreactive T cells inducing autoimmune response in MRL/lpr mice.[34] However, CD138 expression in CD3+ T cells plays a key role in autoimmune response by initiating and promoting the progression of lupus in MRL/lpr mice. CD138 expression in CD3+ T cells significantly promotes the activation of CD3+ T cells in MRL/lpr mice, accelerating the autoimmune response in MRL/lpr mice.[41] Recent studies have found that CD138+ T cells could strikingly contribute to the formation of plasma cells from B cells of MRL/lpr mice.[33 34]

In the present study, we observed significantly reduced production of ANA and anti-dsDNA antibodies in the serum of celastrol-treated MRL/lpr mice. Furthermore, celastrol treatment significantly prevented CD138+ T cell accumulation by specifically promoting apoptosis of CD138+ T cells in MRL/lpr mice. Moreover, our results showed that oral administration of celastrol significantly suppressed the differentiation of CD3+ T cells to Tcm cells in MRL/lpr mice. Interestingly, a previous study reported that the CD138+ T cells contain a large amount of Tcm cells.[34] Our results demonstrated that celastrol prevented T cell-related autoimmune response by promoting CD138+ T cell apoptosis and reduced differentiation of T cells into Tcm cells in MRL/lpr mice. In contrast, prednisone had significant effects on both CD138+ and CD138− T cells and promoted both CD138+ and CD138− T cell apoptosis by increasing apoptosis levels of both CD138+ and CD138− T cells. Different from CD138+ T cells inducing autoimmune response in MRL/lpr mice,[34] CD138− T cells have large amounts of normal T cells. This indicated that celastrol may have an advantage over prednisone in suppressing autoimmune response and simultaneously not suppressing normal immune response.

DN T cells in MRL/lpr mice are strongly cytotoxic and overexpress FasL, which results in autoimmune injuries of multiple tissues that express small amounts of Fas receptor,[30 42] and subsequent exposure of autoantigens to the immune system initiates the autoimmune response. However, CD138 expression could significantly increase FasL expression in DN T cells.[41] CD138 expression will significantly prevent the apoptosis of CD3+ T cells and significantly contribute to the accumulation of DN T cells in MRL/lpr mice.[41] Celastrol significantly prevented DN T cell accumulation in MRL/lpr mice and strikingly reduced CD138+ cell accumulation in DN T cells. Our study demonstrated that celastrol could prevent the autoimmune response by reducing CD138+ cell frequency in DN T cells and subsequently decrease DN T cell accumulation to avoid tissue injury and self-antigen exposure induced by overexpressed FasL in DN T cells.

Immune complexes induce the release of multiple cytokines in the serum of MRL/lpr mice to promote and amplify in vivo inflammation of autoimmune response in MRL/lpr mice.[35 43] In the present study, we observed that

celastrol significantly reduced the production of antibody subsets, including total IgG, IgG_1 and IgG_{2b}, and reduced the serum levels of multiple cytokines, such as TNF, IFN-γ and IL-6, to inhibit the progression of inflammation in MRL/lpr mice. Several studies have shown that T cell polarisation in SLE manifests from Th1 to Th2 cells, and IFN-α promotes activated B cell differentiation into plasma cells, which plays an essential role in SLE disease progression.[44 45] However, our results showed that the frequency of Th1 cells but not Th2 cells in CD4+ T cells of MRL/lpr mice was significantly increased in addition to increased serum levels of IFN-γ. Moreover, the ratio of Th1 to Th2 in MRL/lpr mice was simultaneously increased compared with MRL/MPJ mice. Numerous studies have demonstrated that IFN-γ plays an essential role in the development of lupus in MRL/lpr mice.[43 46–48] IFN-γ significantly promotes the proliferation and accumulation of DN T cells and significantly increases the expression of FasL on the surface of DN T cells in lupus mice.[47 48] Previous in vivo studies have also shown that the frequency of Th1 cells in Fas-deficient lupus mice, but not Th2, was significantly increased.[39 49] Furthermore, studies have also suggested that IFN-γ and Th1 cells may be closely associated with lupus development and tissue injury in MRL/lpr mice.[47 50 51] IFN-γ is required for the toll-like receptor 7 (TLR7)-promoted development of autoreactive B cells.[50] Our results demonstrated that celastrol could significantly prevent IFN-γ expression and reduce Th1 cell frequency in CD4+ T cells in MRL/lpr mice in addition to decreasing serum IFN-γ levels.

TNF-alpha, a proinflammatory cytokine, plays a contradictory role in SLE. Increased TNF levels in the serum, kidneys and skin samples of patients with SLE as well as in SLE murine models play an inflammatory role in SLE and promote organ injury.[52 53] However, TNF is also a cytokine that induces apoptosis in cytotoxic T cells by activating caspase-8 in target cells.[54] It has been reported[55 56] that anti-TNF therapies such as TNF antibodies and soluble TNF receptors are associated with the induction of autoantibodies. Several studies have also proposed that the contribution of TNF in SLE progression is limited; however, increased TNF levels have been shown to contribute to the elimination of DN T cells in SLE murine models resulting in the improvement of lupus symptoms.[54] We observed contradictory results showing that, although serum levels of TNF in MRL/lpr mice were increased, interestingly, the frequency of TNF-producing cells in CD4+ T cells of MRL/lpr mice was significantly reduced compared with MRL/MPJ mice.

In addition to TNF, anti-dsDNA IgG, ANA, multiple cytokines including IFN-γ, IL-6 and IL-12, and multiple antibody subsets levels in the serum were also significantly increased compared with MRL/lpr mice. This suggests that the in vivo environments in MRL/lpr mice are characterised by inflammation and ongoing development of autoimmune responses, leading to the elevated TNF levels in the serum of MRL/lpr mice. However, the TNF+CD4+ T cell frequency in CD4+ T cells of MRL/MPJ mice was induced after 5-hour in vitro stimulation of splenocytes with anti-CD3 antibody compared with vehicle-treated mice. This indicated that TNF+CD4+ T cells were relatively reduced in CD4+ T cells of MRL/lpr, although the serum levels of TNF were elevated compared with MRL/MPJ mice.

Previous studies have reported that TNF blockade therapy alone could result in the induction of ANA and anti-dsDNA despite the frequency and clinical characteristics of anti-TNF-induced lupus (ATIL) varying between the different drugs.[55] In the present study, both celastrol and prednisone significantly reduced serum TNF levels and TNF-producing cell frequencies in CD4+ T cells of MRL/lpr mice. However, celastrol and prednisone administration did not result in ATIL symptoms in MRL/lpr mice. In addition to TNF, celastrol and prednisone could also significantly reduce Th1 cell frequency in CD4+ T cells and decrease serum IFN-γ levels in MRL/lpr mice. Contrary to the contradictory role of TNF, IFN-γ significantly promotes the proliferation and accumulation of DN T cells[47 48] and initiates the development of autoimmune response in lupus.[47 50 51] We speculate that this phenomenon may be related to the effects of celastrol and prednisone, which reduce both IFN-γ levels in the serum and the frequency of IFN-γ+CD4+ T cells within the CD4+ T cell population. These effects could contribute to the prevention of the occurrence of ATIL in MRL/lpr mice.

B cells play a central role in the adaptive immune response. It is believed that autoreactive B cells in SLE can further differentiate into abnormal plasma cells that secrete autoantibodies after activation by self-antigen and autoreactive T cells.[57–60] However, the mechanism by which autoreactive B cells escape negative selection in SLE is still unclear. It is thought that Fas deficiency may result in the failure of apoptosis in autoreactive B cells in SLE.[61 62] After self-antigen exposure, autoreactive B cells would be activated by self-antigens. MHC-II with self-antigens expressed in activated autoreactive B cells interacts with autoreactive CD4+ T cells and activates autoreactive T cells.[58 63] In addition, activated autoreactive B cells will further develop in GC and then differentiate into plasma cells secreting autoantibodies with the help of autoreactive CD4+ T cells.[34 58 63] Our results showed that celastrol strikingly inhibited the activation of B cells and significantly reduced GC B cell frequencies in B cells of MRL/lpr mice. This in turn contributes to the reduced number of plasma cells to prevent B cell-related immune response.

CONCLUSION

We demonstrated that celastrol had a therapeutic effect on lupus in MRL/lpr mice. Celastrol suppressed inflammation to prevent the development and amplification of autoimmune response in lupus. Furthermore, celastrol inhibited the accumulation of DN and CD138+ T cells in MRL/lpr mice by specifically increasing apoptosis levels

of CD138+ T cells. Celastrol treatment also prevented plasma cell generation in MRL/lpr mice by reducing activated and germinal B cell frequency in B cells. The diminished generation of plasma cells from B cells and the alleviated accumulation of CD138+ T cells in MRL/lpr mice treated with celastrol collectively contributed to the reduction in autoantibody secretion.

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