ARTICLE
Journal of Cellular Biochemistry 112:2376–2382 (2011)

Journal of Cellular Biochemistry

Amelioration of Systemic Lupus Erythematosus by Withangulatin A in MRL/lpr Mice

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ABSTRACT

We have previously reported the anti-inflammatory potential and the possible underlying mechanisms of Withangulatin A (WA), which is an active component isolated from *Physalis angulata* L. Here, we demonstrated that WA might improve the life quality, as well as reduced the accumulation of proteinuria symptoms and levels of anti-double-stranded DNA antibodies in MRL/lpr mice. Moreover, WA could improve renal histopathologic characteristics of MRL/lpr mice. Intriguingly, expression of B cell-activating factor (BAFF), BAFF-R and related gene in the spleen were significantly reduced in 10 mg/kg WA-treated mice compared with that in 5 mg/kg WA-treated mice and untreated mice. These findings indicate that WA might have a pleiotropic therapeutic effect through their immunosuppression via inhibiting BAFF signaling, which suggest a potential application of this active constituent in the treatment of SLE. J. Cell. Biochem. 112: 2376–2382, 2011. © 2011 Wiley-Liss, Inc.

KEY WORDS: WITHANGULATIN A; SYSTEMIC LUPUS ERYTHEMATOSUS; BAFF; MRL/LPR MICE

MRL/lpr mice spontaneously develop a lupus like syndrome that can affect many organs, which the murine cutaneous lesions resemble human systemic lupus erythematosus (SLE) [Andrews et al., 1978]. Recent clinical studies have showed that these mice develop high levels of autoantibodies, including anti-DNA antibodies, associated with immune-complex-mediated glomerulonephritis and vasculitis [Eisenberg et al., 1994; Hahn, 1998]. Because of the resemblance between the murine and human diseases, MRL/lpr mice have been used extensively to attempt to determine SLE etiology and evaluate therapies. Indeed, MRL/lpr mice provide an attractive model because their syndrome is spontaneous, predictable, and rapid, it exhibits the characteristic multifaceted tissue destruction and sexual dimorphism, and its severity varies with the individual.

Th1 cytokines, including IFN-γ, are present in the tissues of patients severely affected with SLE. IFN-γ production by peripheral-blood cells and in kidneys of patients with severe lupus glomerulonephritis was higher than that of patients with milder renal disease. Paradoxically, the Th2 cytokine, IL-10, has also been associated with the lupus disease in MRL/lpr mice [Prud'homme et al., 1995]. Likewise, patients with lupus produce large amounts of IL-10, and its level correlates with disease activity [Houssiau et al., 1995; Llorente et al., 1995].

Overproduction of BAFF in transgenic mice leads to an enlarged spleen, increased number of mature B cells, and overt manifestation of SLE [Batten et al., 2000]. Elevated BAFF levels have been detected in the serum of SLE patients, indicating a role for BAFF in these pathologies. Although BAFF is a key B-cell survival factor essential for autoantibody production under autoimmune conditions, it has also been shown to costimulate T cells [Ng et al., 2004]. Despite the incompletely understanding pathogenesis of SLE in details, extensive efforts have focused on the development of effective drug therapy with limited side effects.

Immunosuppressive drugs have shown beneficial effects in the treatment of SLE-associated autoimmune diseases, but have a limited effect on lymphoproliferation, as their prolonged use is

Lijuan Sun and Lin Zhou contributed equally to this work.

Grant sponsor: China 111 Project; Grant number: B07023; Grant sponsor: The National Natural Science Fund Project; Grant number: 30901368; Grant sponsor: Shanghai Youth Science and Technology Development Project; Grant number: 09QA1407400.

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Received 14 January 2011; Accepted 19 April 2011 • DOI 10.1002/jcb.23160 • © 2011 Wiley-Liss, Inc.
Published online 25 April 2011 in Wiley Online Library (wileyonlinelibrary.com).

fraught with complications. *Physalis angulata* L., a traditional Chinese herb medicine, its fruits are edible as health wine by many in China. This herb medicine is widely used in the treatment of various inflammatory disorders, especially for rheumatoid arthritis and dermatitis [Caceres et al., 1995; Ankrah et al., 2003]. Here we show that WA, isolated out from *Physalis angulata* L., exerts pleiotropic therapeutic effect. In our previous work, we reported the mechanism of anti-inflammatory [Sun et al., 2010] and immunosuppressive effect [Sun et al., 2011] of WA, which might protect against autoimmune disorder diseases. This study showed WA could improve the life quality and renal histopathologic characteristics, as well as reduce the accumulation of proteinuria symptoms and levels of anti-double-stranded DNA antibodies in MRL/lpr mice, which suggested a potential application of this active constituent in the treatment of SLE.

MATERIALS AND METHODS

MICE AND MATERIALS

Fifteen-week-old female MRL/lpr mice were purchased from Shanghai Experimental Animals Center (License number: SCXK 2007-0002), bred and maintained in the Laboratory of Animal Experiments at Shanghai Jiao Tong University School of Medicine. All mice were accustomed in our SPF standards animal laboratory for 1 week before experiments started. Animal experiments were conformed to the Guide for the Care and the Use of Laboratory Animals (1996). Withangulatin A (WA) was kindly supplied by Prof. Lihong Hu Research Group, Shanghai Innovative Research Center of Traditional Chinese Medicine, Shanghai Institute of Materia Medica, Chinese Academy of Sciences. For the current experiments, the agent was dissolved in saline with 0.5% Tween-80. Mice were divided into the following five groups, according to treatment: (1) WA at 20 mg/kg, (2) WA at 10 mg/kg, (3) WA at 5 mg/kg, (4) cyclophsophamide (CTX) at 20 mg/kg, and (5) vehicle solution ($n = 7$ per group). Treatment was administered by oral gavage four times per week up to 24 weeks of age.

LABORATORY ASSESSMENTS

All mice in each group were placed overnight in a Nalgene metabolic cage to collect urine, and urinary protein levels were determined with Multistix 10 SG and analyzed by Clinitek Status analyzer (Bayer Healthcare) in each group. After the mice were extinguished, the extent of lymphadenopathy and splenomegaly was assessed by determining the weight of the swollen lymph nodes and the spleen respectively. Serum anti-double-stranded DNA (anti-dsDNA) antibodies were analyzed by indirect immunofluorescence kit (Shibayagi, Gunma, Japan). ELISA was used to quantify serum BAFF as previously described with modification assay [Zhang et al., 2001].

HISTOPATHOLOGY AND IMMUNOHISTOPATHOLOGY OF THE KIDNEYS

The kidneys were fixed in 10% formalin for 24 h at 4°C. Paraffin sections (4 μm) were stained with hematoxylin and eosin. The severity of glomerulonephritis was evaluated in a blinded manner by histologic examination of the sectioned kidneys, and the results were further confirmed by the statistically significant difference in the crescent formation rate. A total of 100 glomerular cross-sections (GCS) per kidney were examined, and the crescent formation rate was determined (crescent-formed glomeruli/GCS). Changes were scored on a semiquantitative scale of 0–3, where 0 = normal (35–40 cells/GCS), 1 = mild (few lesions, with slight proliferative changes and mild hypercellularity [41–50 cells/GCS] and/or minor exudation), 2 = moderate (moderate hypercellularity [51–60 cells/GCS], including segmental and/or diffuse proliferative changes, hyalinosis, and/or moderate exudates), and 3 = severe (segmental or global sclerosis and/or severe hypercellularity [>60 cells/GCS], necrosis, crescent formation, and/or heavy exudation). For immunohistochemical, BAFF or BAFF-R of spleen was detected using rabbit anti-mouse BAFF or BAFF-R antibody (Abcam, UK), followed by a universal secondary antibody. After one wash, the avidin–peroxidase reagent and then 3,3′-diaminobenzidine tetrahydrochloride (DAB) as peroxidase substrate were incubated for 10 min each with an intermediate wash. After color development, the sections were counterstained with hematoxylin and mounted in glycerol medium. The stained tissue sections were photographed with a Zeiss Universal microscope.

REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION

We assessed the transcription levels of BAFF, BAFF-R, a proliferation-inducing ligand (APRIL), IFN-γ, and IL-10 mRNA, relative to the levels of GADPH in the spleen from MRL/lpr mice. Total RNA samples from mice spleen were prepared and reversed transcribed with Revert Aid™ First Stand cDNA Synthesis kit (Fermentas, EU). Quantitative real-time PCR and melt-curve analyses were performed with the SYBR Green Realtime PCR kit (TOYOBO, Japan) and an iCycler PCR machine (Bio-Rad, Hercules, CA). The following conditions were used: 95°C for 2 min, 40 cycles of 95°C for 30 s and 60°C for 30 s, followed by 72°C for 30 s, with a final extension step at 72°C for 10 min. The threshold cycle (C_T) of gene products was determined and set to the loglinear range of the amplification curve and kept constantly. Relative expressions were calculated with normalization to GADPH values by using the comparative C_T method, where $\text{fold difference} = 2^{-(C_T \text{ of experimental gene} - C_T \text{ of GADPH})} = 2^{-\Delta C_T}$. The sequences of the primers were designed to span at least one intron.

GADPH: Sense 5′-AGGTCGGTGTGAACGGATT-3′, antisense 5′-CTCCTGGAAGATGGTGATGG-3′; BAFF: Sense 5′-TGCCTTGGAG-GAGAAAGAGA-3′, antisense 5′-GGAATTGTTGGGCAGTGTTT-3′; BAFF-R: Sense 5′-GGAAAATGTCTTTGTACCCTC-3′, antisense 5′-CTATTGCTCTGGGCCAGCTGT-3′; APRIL: Sense 5′-GATGTGG-CAACCAGTACTTAGG-3′, antisense 5′-TATTGTAGGCACGGTCAG-GAT-3′; IFN-γ: Sense 5′-AAGCGTCATTGAATCACACC-3′, antisense 5′-CGAATCAGCAGCGACTCCTT-3′; IL-10: Sense 5′-ACCTGGTA-G.AAGTGATGCCCCAGGCA-3′, antisense 5′-CTATGCAGTTGATG-AAGATGTCAAA-3′.

STATISTICAL ANALYSIS

Data were expressed as means ± SD. One-way ANOVA was used to determine significance between groups where appropriate $P < 0.05$ was considered significant.

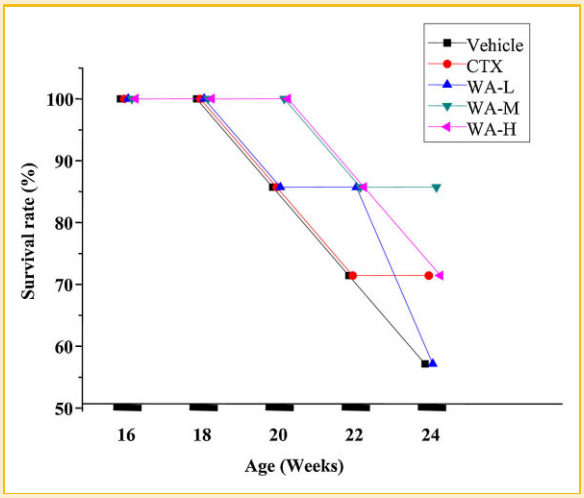

Fig. 1. Effect of WA on survival rate of MRL/lpr mice. Prolonged survival of WA-treated MRL/lpr mice. The cumulative survival of female mice treated with 5 mg/kg WA, 10 mg/kg WA, 20 mg/kg WA, 20 mg/kg CTX, or vehicle solution was monitored daily (n = 7 per group).

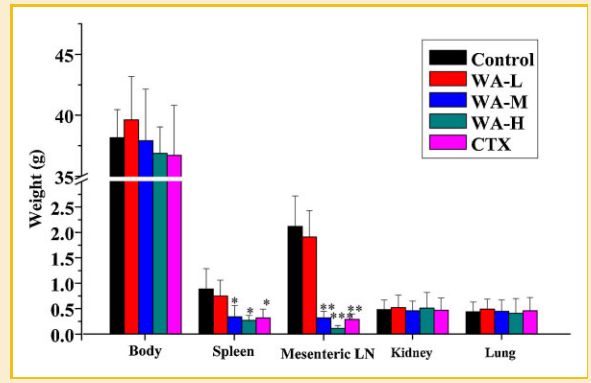

Fig. 2. Changes of body weight, spleen, mesenteric lymph nodes, kidney, and lung in the female MRL/lpr mice at 24 weeks of age. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with control group.

RESULTS

PROLONGED SURVIVAL OF WA-TREATED MRL/lpr MICE

In order to examine the effect of WA on the MRL/lpr mice, we monitored their survival. Mice treated with 10 mg/kg of WA showed better survival rate compared with other four groups, treated with 5 mg/kg, 20 mg/kg of WA, 20 mg/kg of CTX, and vehicle solution; the survival rate of WA-high group (20 mg/kg) was similar to positive control group (CTX, 20 mg/kg) (Fig. 1). The difference in survival rates between five groups was distinct. The fact that improvement in life quality of the treated mice was observed since 20 weeks of age might be a reflection of the prolonged survival rate of these mice.

REDUCED THE SPLENOMEGALY

To determine the effects of WA treatment on splenomegaly in the MRL/lpr mice, the total body, spleen, and mesenteric lymph nodes (LN) weight were determined in five groups of mice at 24 weeks. Splenomegaly in the 10 and 20 mg/kg WA-treated mice was reduced, as the weight of spleen and mesenteric LN dropped significantly ($P < 0.05$), which little difference compared with CTX group, and the weight of body, kidney, and lung were not significant reduced (Fig. 2).

REDUCED PROTEINURIA AND ANTI-dsDNA ANTIBODIES

To investigate whether WA alters typical clinical symptoms of SLE, we evaluated the levels of proteinuria and anti-dsDNA antibodies. Proteinuria was significantly reduced in 10 mg/kg WA-treated mice (40.12 ± 13.03 mg/dl) compared with control group (231.15 ± 11.31 mg/dl) at 24 weeks (Fig. 3A). Levels of anti-dsDNA antibody in serum were 800 ± 153 U/ml in 10 mg/kg WA-treated mice, 1,100 ± 222 U/ml in 20 mg/kg WA-treated mice, and 3,497 ± 123 U/ml in the untreated mice at 24 weeks (Fig. 3B). Thus, levels of proteinuria and anti-dsDNA antibodies were significantly reduced in 10 and 20 mg/kg WA-treated mice compared with the mice administered vehicle solution.

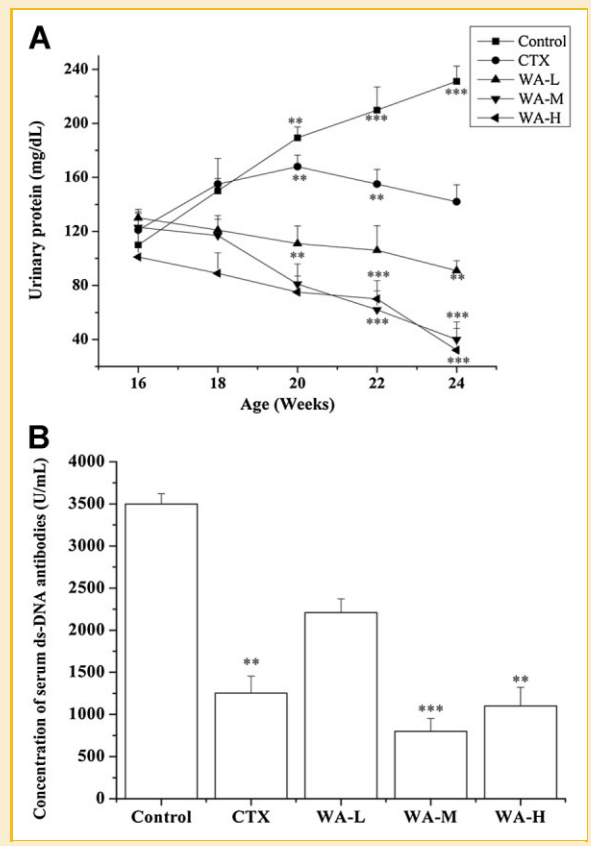

Fig. 3. Levels of urinary protein and anti-dsDNA antibodies in the five groups of MRL/lpr mice. The urinary protein was determined in the mice administered vehicle solution, 5 mg/kg WA, 10 mg/kg WA, 20 mg/kg WA, and 20 mg/kg CTX in five different time point (A). The anti-dsDNA antibodies were determined in the MRL/lpr mice at 24 weeks of age (B). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with control group.

REDUCTION OF SERUM BAFF BY WA-TREATED MICE

The concentrations of serum BAFF was significantly reduced from the WA-treated mice compared with the untreated mice (2.91 ± 0.62 ng/ml in 10 mg/kg WA-treated mice and 3.72 ± 0.64 ng/ml in 20 mg/kg WA-treated mice vs. 11.22 ± 0.52 ng/ml in the untreated mice) (Fig. 6A).

AMELIORATED GLOMERULONEPHRITIS BY WA IN MRL/LPR MICE

The kidneys of the 5 mg/kg WA-treated mice and the untreated mice at 24 weeks showed typical glomerulonephritis, characterized by enlarged glomeruli, proliferation of glomerular cells, the expansion of the mesangial extracellular matrix (ECM), infiltrating lymphocytes, neutrophils, plasma cells, and a small amount of macrophages, degeneration of renal tubular epithelial cells in some regions, vasodilator congestion significantly (Fig. 4A,F). In contrast, mice treated with 20 mg/kg WA and 20 mg/kg CTX showed a lesser degree of glomerulonephritis, varying degree of focal glomerular cell proliferation, only a slight increase in the ECM and degeneration of renal tubular epithelial cells, occasional inflammatory cells, vasodilator congestion slightly, and the therapeutic effect of 20 mg/kg WA group was even better than CTX positive control group (Fig. 4D,I,E,J). There was a substantial reduction in the number of inflammatory cells in the interstitium, periglomeruli, and intraglomeruli of the kidneys of the 20 mg/kg WA-treated mice compared with those of the 5 mg/kg WA-treated mice and the untreated mice. There was significant difference in the kidneys scores between the five groups on a semiquantitative scale (Fig. 4K).

REDUCTION IN THE EXPRESSION OF PROTEIN AND mRNA FOR BAFF AND BAFF-R, AS WELL AS OTHER RELATIVE GENE IN SPLEEN FROM WA-TREATED MICE

We detected the expressions of mRNA for BAFF and its one of important BAFF receptors, BAFF-R (Fig. 5), there was a significant decrease of BAFF and BAFF-R mRNA expressions after the treatment of WA (BAFF/GADPH 0.031 ± 0.0046 in 10 mg/kg WA-treated mice and 0.0037 ± 0.0021 in 20 mg/kg WA-treated mice vs. 0.022 ± 0.0069 in CTX-treated mice and 0.059 ± 0.0083 in the untreated mice, BAFF-R/GADPH 0.039 ± 0.014 in 10 mg/kg WA-treated mice and 0.040 ± 0.018 in 20 mg/kg WA-treated mice vs. 0.031 ± 0.0080 in CTX-treated mice and 0.20 ± 0.073 in the untreated mice), implicating a potential role for BAFF in ameliorating of SLE by WA.

We next sought to determine the role of APRIL, IFN-γ, and IL-10 mRNA which mediated BAFF overproduction in spleen. Expression

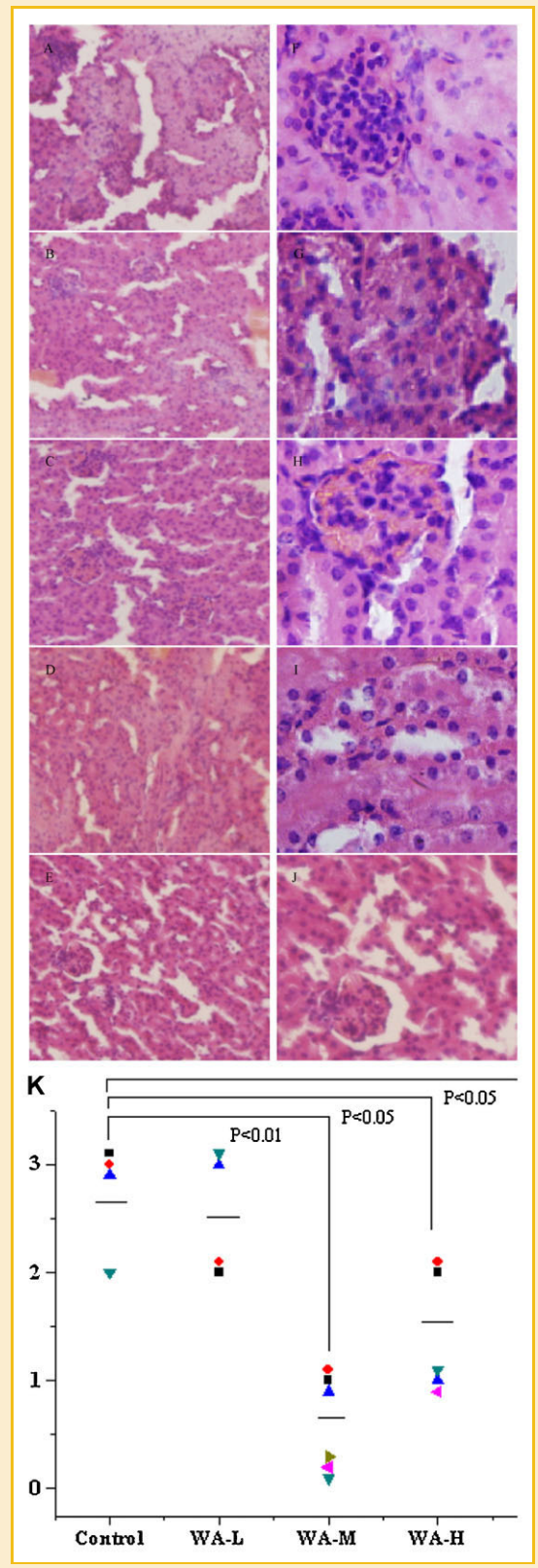

Fig. 4. Histopathologic features of kidneys in MRL/lpr mice. Altered histopathologic features of kidneys in WA-treated MRL/lpr mice. The kidneys from untreated (A and F, respectively), 5 mg/kg WA treated (B and G, respectively), 10 mg/kg WA treated (C and H, respectively), 20 mg/kg WA treated (D and I, respectively), and 20 mg/kg CTX treated (E and J, respectively) 24-week-old MRL/lpr mice were assessed by immunohistopathology (stained with hematoxylin and eosin; original magnification ×200 in A–E, ×400 in F–J). The severity score for kidney sections from untreated, 5 mg/kg, 10 mg/kg, 20 mg/kg WA treated, and 20 mg/kg CTX-treated MRL/lpr mice (K).

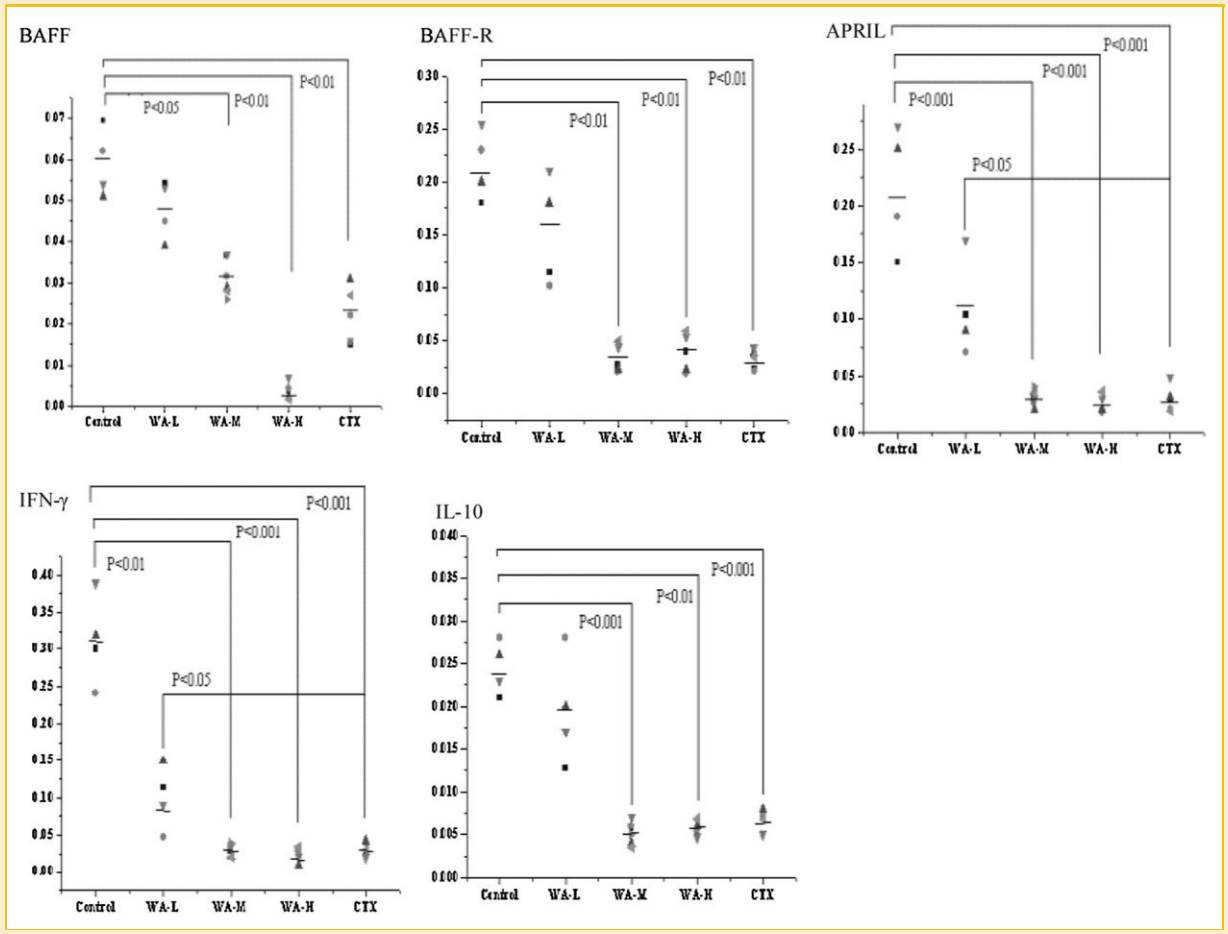

Fig. 5. BAFF, BAFF-R, APRIL, IFN-γ, and IL-10 mRNA expression in the spleen from MRL/lpr mice. The mRNA expression of BAFF, BAFF-R, APRIL, IFN-γ, and IL-10 in the spleen sections was compared from the mice administered vehicle solution, 5 mg/kg WA, 10 mg/kg WA, 20 mg/kg WA, or 20 mg/kg CTX (n = 7 per group). The levels of mRNA were analyzed by Real-time PCR; GADPH was shown as the loading control. Each data point represented the mean of individual mouse in three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with control group.

of APRIL, IFN-γ, and IL-10 mRNA between five groups was also reduced varying degree in spleen from WA-treated mice compared with the untreated mice (Fig. 5).

The proportional area of staining positive for BAFF and BAFF-R was also significantly reduced in 10 mg/kg WA-treated mice compared with that in 5 mg/kg WA-treated mice and the untreated mice (Fig. 6B,C).

DISCUSSION

In this study we have demonstrated that WA significantly attenuated the disease phenotype of MRL/lpr mice. There were significant differences in the urinary protein and anti-dsDNA antibodies (Fig. 3) between the mice treated with WA and untreated mice. Light microscopic examination of the kidney tissues showed protection against attenuating severity of glomerulonephritis correlates well with reduced ECM of the glomeruli and the number of infiltrating leukocytes (macrophages and T cells) (Fig. 4). In conjunction with these clinic and histopathologic improvements, we speculated that WA could prolong the survival rate of MRL/lpr mice, even though the treatment was initiated in the advanced stage of the disease.

In normal spleen tissue, the expression of BAFF and BAFF receptor is low, and their levels increase in conjunction with the severity of glomerulonephritis [Kayagaki et al., 2002]. Consistent with the well-recognized function of BAFF as a key survival factor for B-cell maturation and activation [Mackay and Browning, 2002], our immunohistochemical study revealed that the proportional area of spleen expressing BAFF and BAFF receptor was significantly reduced in 10 and 20 mg/kg WA-treated mice (Fig. 6C) compared with that in the untreated mice, as well as the equivalent role of CTX. In addition, increasingly recognized central role of B cells in the immunopathogenesis of SLE, as demonstrated by the clinical benefits of B cell-targeted therapies for these diseases [Eisenberg and Albert, 2006], a significant reduction in the expression of protein and mRNA for BAFF and BAFF receptor in the spleen of the 20 mg/kg WA-treated mice was the further evidence that WA might inhibit the BAFF signaling pathway in the MRL/lpr mice. Although the pathogenic role of SLE remains to be further established, there is

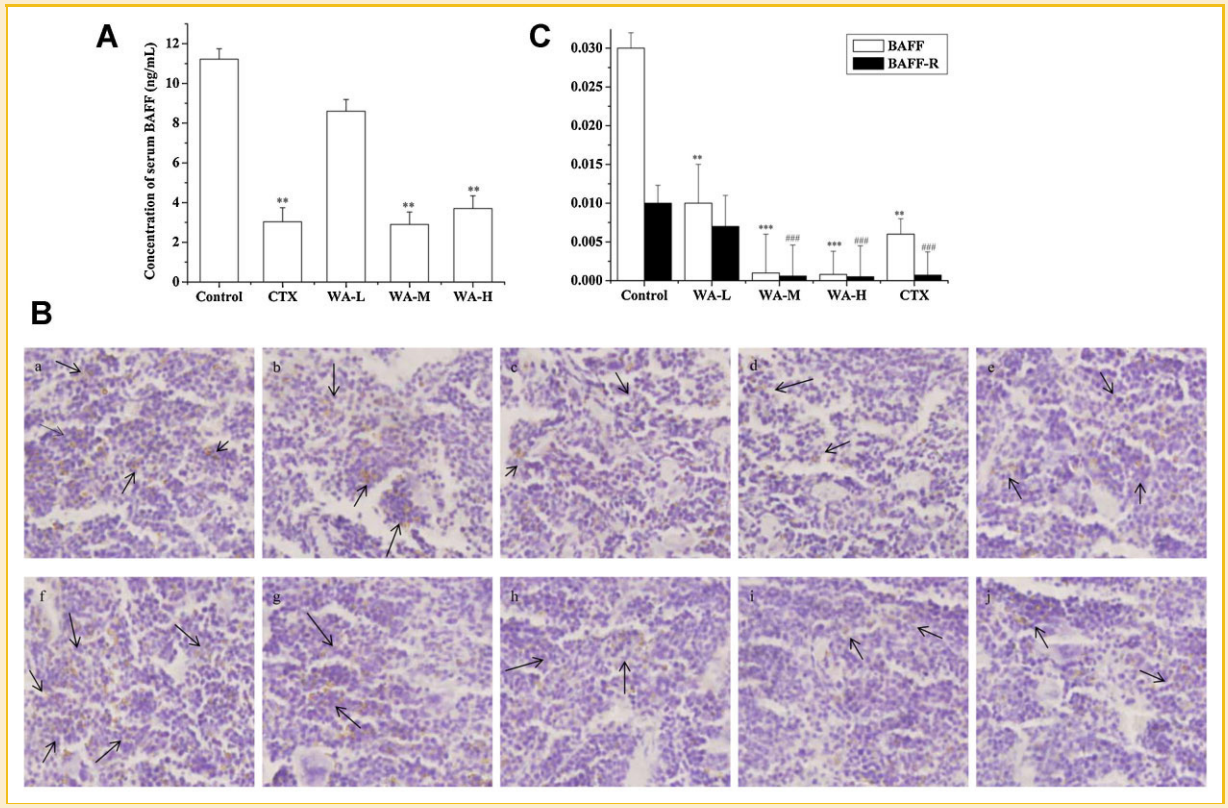

Fig. 6. Levels of serum BAFF in the five groups of MRL/lpr mice. The serum concentrations of BAFF were determined by ELISA in the mice administered vehicle solution, 5 mg/kg WA, 10 mg/kg WA, 20 mg/kg WA, and 20 mg/kg CTX (A). BAFF and BAFF-R protein expression in the spleen from MRL/lpr mice. Immunohistochemical staining for BAFF and BAFF-R was performed in the spleen from untreated (a and f, respectively), 5 mg/kg WA treated (b and g, respectively), 10 mg/kg WA treated (c and h, respectively), 20 mg/kg WA treated (d and i, respectively), and 20 mg/kg CTX treated (e and j, respectively). The proportional area of spleen staining (bar indicates the mean) for BAFF and BAFF-R (C).

strong evidence that BAFF as a key target potentially applicable for the treatment of patients with SLE.

Intriguingly, it has been demonstrated that WA inhibits mice T lymphocytes proliferation stimulated with LPS and pro-inflammation cytokines, including IL-2, IFN-γ, and IL-6 in vitro [Sun et al., 2010], as well as WA significantly suppressed the over-proliferation of ConA-induced T lymphocytes and markedly reduced Th1-type cytokines and increased Th2-type cytokines to obtain Th1/Th2 balance [Sun et al., 2011]. WA also attenuated the lymphadenopathy in MRL/lpr mice. The weight of the Mesenteric LN and spleen (Fig. 2) were significantly different between the five groups. Mishra et al. [2003] and Reilly et al. [2004] demonstrated that treatment of MRL/lpr mice subcutaneously with trichostatin and parenterally with SAHA resulted in reduced overall kidney pathology, respectively. Trichostatin A decreased levels of IFN-γ, IL-12p40, IL-6, and IL-10 proteins in splenocytes from MRL/lpr mice in vivo [Houssiau et al., 1995]. These cytokines have the potential to contribute to inflammation and pathologic changes in the kidney. Moreover, the ability of IFN-γ and IL-10 [Nardelli et al., 2001] up-regulate BAFF expression suggests that inflammatory signals might in part regulate the expression of BAFF. BAFF shares significant homology with APRIL, which stimulates tumor cell growth as well as proliferation of primary lymphocytes [Hahne et al., 1998]. Additional studies are ongoing to explore the impact of WA on autoimmunity by looking at kidney, cytokines, and autoantibody production. Consistent with this idea, the expression of IFN-γ, IL-10, and APRIL was significantly suppressed in 10 mg/kg WA-treated mice compared with that in the other groups. WA might preferentially suppress IFN-γ, IL-10, and APRIL production by inhibiting T cell proliferation. In any case, these data again support that WA has a direct immunomodulatory effect on immune cells, and this is attributable, at least partly, to the amelioration of SLE in the MRL/lpr mice.

These in vivo and in vitro data suggest that WA exerts a direct effect on immune cells, in addition to its inhibition of BAFF signaling. Thus, WA might have synergistic effects with the immunosuppressive drugs cyclosporin A and FK506, which are potential inhibitors of T cell receptor-mediated IL-2 production [Ho et al., 1996]. Thus, it seems that this immunosuppression via inhibition of BAFF signaling was the mechanism of ameliorating SLE by WA.

In conclusion, the present study provides the first direct in vivo evidence of the therapeutic efficacy of WA in the treatment of SLE in MRL/lpr mice. WA significantly attenuates the glomerulonephritis, the lymphadenopathy, and the immunologic disorder in 24-week-old mice, as well as prolonged the survival rate of the MRL/lpr mice. Because of its combined effects through the above-described mechanisms, and there perhaps are more fundamental epigenetic differences have yet to be determined between SLE patients and

normal individuals, further studies are required to explore mechanistic questions and gain further insights into this class of drugs, WA or combination with another agent(s) might play a role as a novel strategy in the treatment of SLE in humans.

ACKNOWLEDGMENTS

This work was supported by China 111 Project (no. B07023), The National Natural Science Fund Project (no. 30901368), Shanghai Youth Science and Technology Development Project (no. 09QA1407400). We thank Ms. S.F. You for her excellent technical assistance.

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