

Withaferin A, a candidate drug for lupus nephritis, confers renal protection via Pon1-mediated attenuation of oxidative stress

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Abstract. Lupus nephritis (LN) is a severe manifestation of systemic lupus erythematosus (SLE). It is characterized by autoantibody deposition and immune complex formation within the kidney, leading to progressive nephron loss and end-stage renal disease. Suppression of immunology is a cornerstone for managing LN. The present study identified withaferin A (WA) as a promising therapeutic candidate for SLE via connectivity map analysis and validated its efficacy in ameliorating LN in an MRL/lpr mouse model. Candidate drugs for LN were identified via the Connectivity Map database by integrating transcriptomic signatures from GSE135779, GSE162577 and GSE142016. An animal model was established to evaluate the therapeutic efficacy of WA on LN-associated inflammation and splenic dysfunction, followed by RNA-sequencing analysis. To elucidate the mechanistic role of PON1 in WA-mediated protection, siRNA-mediated knockdown and plasmid-driven overexpression were performed in HK-2 cells. Furthermore, the direct impact of WA was assessed using isolated primary B cells. WA improved renal function by mitigating splenic immune cell dysregulation and attenuating renal inflammation. Mechanistically, RNA-sequencing analysis and functional validation revealed that WA upregulated paraoxonase 1 (Pon1) expression, which in turn alleviates renal injury by decreasing reactive oxygen species via the peroxisome proliferator-activated receptor signaling pathway. Pon1 activation enhanced cell viability, suppressed inflammatory responses and decreased oxidative stress in HK-2 cells, underscoring its potential as a novel therapeutic target for LN.

Collectively, the findings demonstrate WA is a viable candidate for SLE/LN treatment and Pon1 is a pivotal mediator of its protective effects, thereby providing a dual-strategy insight for clinical intervention.

Introduction

Systemic lupus erythematosus (SLE) is a prototypical chronic autoimmune disease. It is characterized by dysregulated adaptive immunity, leading to systemic inflammation and immune-mediated damage affecting multiple organ systems, including the mucocutaneous, musculoskeletal, hematological and renal systems. Lupus nephritis (LN) is a severe complication of SLE that can progress to glomerulosclerosis, interstitial fibrosis and renal failure in untreated patients (1). Clinical management of LN primarily relies on immunosuppressive therapies, such as corticosteroids (2), cyclophosphamide (3), mycophenolate mofetil/mycophenolic acid (3) and calcineurin inhibitors (4). However, these regimens achieve complete remission in 20–30% of patients after 6 months of standard treatment (5), highlighting a notable unmet therapeutic need. This suboptimal response rate underscores the need for novel pharmacotherapies that target the underlying pathogenic mechanisms of LN.

SLE is driven by a cascade of aberrant immune responses, including hyperactivation of B and T cells, overactive type I IFN signaling and neutrophil dysfunction. The disease is typically associated with the accumulation of endogenous immunogenic chromatin, which activates innate immune sensors via toll-like receptors and simultaneously promotes the expansion of chromatin-reactive B cells (6). This dual mechanism sustains B cell activation and leads to the production of anti-DNA and antinuclear autoantibodies. In SLE, elevated levels of soluble B cell activating factor (BAFF) drive B cell activation and autoantibody production. Consequently, therapeutic strategies targeting BAFF or depleting B cells have demonstrated clinical efficacy by decreasing autoreactive B cell populations. Notably, genetic and functional studies have revealed conserved signaling defects in SLE B cells, marked by dysregulation of key kinases (such as Lyn) (7,8) and phosphatases (such as CD22 and Src homology 2 domain-containing protein tyrosine phosphatase 1) (9). In lupus-prone mouse

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models, loss of these regulatory molecules results in B cell hyperactivation, autoantibody production and lupus-like nephritis (7-9), whereas pharmacological restoration of their expression attenuates disease progression (10,11).

RNA sequencing (RNA-seq) is a tool for profiling gene expression in immune cells, enabling the identification of dysregulated pathways, such as type I IFN and B cell receptor signaling, and the discovery of druggable targets. Single-cell (sc)RNA-seq improves resolution by identifying cell types within a tissue and identifying rare pathogenic subsets, such as IFN-responsive plasmablasts, which may serve as precision medicine endpoints. Recent advances in scRNA-seq enable unbiased characterization of immune cell diversity and revealing potential therapeutic targets (12). For example, Arazi *et al* (12) applied scRNA-seq to identify 21 distinct leukocyte subsets in patients with SLE, including diverse populations of myeloid cells, T, natural killer and B cells. The aforementioned study identified two broadly expressed chemokine receptors as potential therapeutic nodes. Moreover, scRNA-seq enables the reconstruction of functional intercellular networks by resolving gene expression landscapes at single-cell resolution, offering insights into the pathogenic roles of B cells and their microenvironmental crosstalk.

Drug repurposing offers a cost-effective strategy to accelerate drug development while decreasing the financial burden of *de novo* discovery. Moreover, *in silico* analysis of gene expression signatures is effective in identifying new therapeutic applications for existing compounds (13). The Connectivity Map (CMAP; clue.io/) is a platform that links disease transcriptomes with gene perturbations and drug mechanisms of action. The CMAP database has been utilized in drug repurposing for cancer, neurodegeneration and metabolic disorders (14). The present study analyzed transcriptomic profiles of peripheral B lymphocytes and compared them with the CMAP database. As a bioactive steroidal lactone extracted from *Withania somnifera*, Withaferin A (WA) exhibits a broad spectrum of pharmacological properties, most notably anti-inflammatory (15), antioxidant (16), and anticancer activities (17). Its relevance in therapeutic interventions has been increasingly documented, primarily through its ability to target inflammation (15) and oxidation (16). Therefore, characterization of its effects on LN is vital to elucidate its full biomedical value.

Materials and methods

Reagents and antibodies. WA (cat. no. HY-N2065), DMSO (cat. no. HY-Y0320) and lipopolysaccharide (cat. no. HY-D1056) were purchased from MedChemExpress. The antibodies were as follows: Mouse α -smooth muscle actin (SMA; cat. no. ab7817), TNF- α (cat. no. ab183218), IL-1 β (cat. no. ab283818), kidney injury molecule-1 (KIM-1; cat. no. ab47635), paraoxonase 1 (Pon1; cat. no. ab92466), peroxisome proliferator-activated receptor (PPAR)- α (cat. no. ab126285), PPAR- γ (cat. no. ab45036), Bax (cat. no. ab32503), BCL-2 (cat. no. ab182858) and GAPDH (cat. no. ab8245; all Abcam). The mouse anti-nuclear antibody (ANA)/extractable nuclear antigen (ENA) Igs (total A + G + M;

cat. no. 5210) and mouse anti-double-stranded DNA Igs (total A+G+M) ELISA kit (cat. no. 5110) were obtained from Alpha Diagnostic Intl Inc.

CMAP assay. To identify potential therapeutics for LN, scRNA-seq data derived from human peripheral blood were obtained from the Gene Expression Omnibus database. (ncbi.nlm.nih.gov/gds). Differentially expressed genes (DEGs) in B lymphocytes from GSE135779, GSE162577 and GSE142016 were identified by comparing patients with SLE with healthy donors (adjusted $P < 0.05$). To represent the disease-specific expression profile, the top 150 upregulated and top 150 downregulated genes were selected as the gene signature for the CMAP/L1000 database using the CLUE web platform (<https://clue.io/>), based on their rank in $\log_2$ Fold Change magnitude. Connectivity scores were calculated and the highest-ranking compounds from the CMAP/L1000 analysis were selected for further investigation.

Animal model and experimental design. MRL/lpr mice ($n=10$; weight, 27.71 ± 0.86 g) were purchased from Changzhou Cavens Laboratory Animal Co., Ltd. and maintained under specific pathogen-free conditions at the Animal Center of Gansu University of Chinese Medicine (Lanzhou, China). Mice were housed at $22 \pm 2^\circ\text{C}$ and relative humidity of $50 \pm 10\%$, with a 12/12-h light/dark cycle. Female mice (age, 8 weeks) were randomly allocated into two groups ($n=5$ per group). The treatment group received intraperitoneal injection of 2 mg/kg WA dissolved in DMSO and the control group was treated with 2% DMSO once/day. All animals had free access to food and water throughout the study. After 8 weeks of treatment, mice were euthanized by CO_2 asphyxiation with a chamber fill rate of 60% of total volume/min, in accordance with the AVMA Guidelines for the Euthanasia of Animals (18), followed by sample collection for downstream analyses including spleen, kidney and lymph gland. All experimental procedures were approved by the Animal Experimental Ethical Inspection of Gansu University of Chinese Medicine (approval no. SY2024-257) and performed in compliance with institutional guidelines for animal welfare and use.

Renal function. According to the manufacturer's instructions, serum creatinine (cat. no. EIASCR) and urea nitrogen (cat. no. BC1535) were measured using commercial kits from Thermo Fisher Scientific, Inc. and Beijing Solarbio Science & Technology Co., Ltd., respectively. Urinary protein levels were determined using the Pierce™ Bradford Plus Protein Assay kit (cat. no. 23236; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol.

ELISA for ANAs and anti-double-stranded (ds)DNA antibodies. The concentrations of ANA and dsDNA antibodies in mouse serum were quantified using the aforementioned ELISA kits, following the manufacturer's instructions. In brief, samples and controls were incubated at 37°C for 60 min. HRP-conjugated anti-mouse Ig horseradish peroxidase (1:100) and tetramethylbenzidine substrate were added for 30 and 15 min at 37°C , respectively. Absorbance was measured at 450 nm using a microplate reader.

Histology. Tissue samples were fixed in 4% paraformaldehyde prepared in 1X phosphate-buffered saline (pH 7.5) at room temperature overnight, dehydrated through a graded series of ethanol and embedded in paraffin. 3- μ m-thick sections were stained with hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) using commercial staining kits (cat. no. G1120 and G1281 for PAS, respectively; both Solarbio) at room temperature (hematoxylin for 5 min and eosin for 2 min. For PAS staining, sections were oxidized in periodic acid for 15 min, incubated with Schiff's reagent for 15 min in the dark, and counterstained with hematoxylin for 1 min. to evaluate renal structural damage and pathological changes. The pathological changes in the tissue were observed under the light microscope (Zeiss Axio Imager M2) and analyzed by ImageJ (version 1.53, National Institutes of Health). Glomerulosclerosis was assessed using a semi-quantitative scale as follows: 0, normal; 1, mesangial expansion and slight glomerular damage involving <25% of the glomerulus; 2, mild sclerosis involving 25-49% of the glomerulus; 3, moderate sclerosis involving 50-74% of the glomerulus; and 4, severe sclerosis involving $\geq$ 75% of the glomerulus (19).

Immunofluorescence. For the detection of glomerular immune complex deposition, kidney tissues were embedded in OCT compound and frozen at -20°C. Frozen sections with a thickness of 5 μ m were prepared. Following being blocking with 5% bovine serum albumin (HY-D0842, MedChemExpress) for 1 h at room temperature, the sections were incubated with primary antibodies, including rabbit anti-complement C3 (1:200; cat. no. ab97462; Abcam), overnight at 4°C. Sections were incubated with secondary antibodies, such as Cy5-conjugated goat anti-mouse IgG (H+L; 1:500; cat. no. 771421ES; Yeasen) or Alexa Fluor-labeled antibodies (both 1:500; cat. no. A-11012; Thermo Fisher Scientific), for 1 h at room temperature. Images were captured using a microscope (LSM 800, Zeiss) and analyzed using ImageJ (version 1.53, National IH). Semiquantitative analysis of glomerular complement C3 deposition was performed as follows: 0, no staining; 1, barely detectable at high magnification; 2, moderately visible; and 3, strongly and clearly visible (20).

Immunohistochemistry (IHC). Renal α -SMA expression was evaluated via IHC. Briefly, kidney tissues were fixed in 4% paraformaldehyde for 24 h at 4°C and embedded in paraffin. Sections were cut at a thickness of 3-5 μ m. Following deparaffinization in xylene and rehydration through a descending alcohol series, antigen retrieval was performed by heating in 10 mM citrate buffer, pH 6.0 at 95°C for 15 min. To block endogenous peroxidase activity, sections were treated with 3% H₂O₂ for 10 min at room temperature. Following blocking with 5% normal goat serum (Beijing Solarbio) for 1 h at room temperature, sections were incubated with primary anti- α -SMA antibodies (1:2,000; cat. no. ab124964; Abcam) overnight at 4°C. Following four washes in PBS, sections were incubated with HRP-conjugated secondary antibodies (1:500; cat. no. GB23303; Servicebio) for 1 h at room temperature. The signal was developed using a DAB substrate kit and counterstained with hematoxylin for 2 min at room temperature. Images were acquired using the light microscope (Zeiss Axio

Imager M2). Positive staining area was measured using ImageJ software (version 1.53).

RNA-seq. To prepare RNA-seq samples, kidneys were harvested from the SLE model mice following treatment with either DMSO or WA. All tissues were immediately snap-frozen in liquid nitrogen and stored at -80°C. Total RNA was extracted using the GeneJET RNA Purification kit (cat. no. K07311 Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions, followed by DNase I treatment to eliminate genomic DNA contamination. RNA quality and quantity were assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies) to ensure an RNA Integrity Number >7.0 prior to cDNA library construction. Sequencing was performed on an DNBSEQ-T7 platform (MGI, Inc.) by Sangon Biotech Co., Ltd. The sequencing is performed in 150 bp paired end using DNBSEQ DNB Reagent Kit (cat. no. 1000028453; MGI Inc.). The loading concentration of the final library is 18.6 pM. Transcript abundance was quantified using the transcripts per million methods, which normalizes gene expression based on exon models, enabling cross-gene and cross-experiment comparability. Differential expression analysis was performed using the DESeq2 R package (v1.12.4) (<https://www.r-project.org>), applying a significance threshold of $|\text{fold-change}| > 2$ and an adjusted P-value of <0.05. Based on the differentially expressed genes (DEGs), hierarchical clustering and heatmap visualization were performed using R software gplots package (2.17.0). Functional annotation of DEGs was performed using Gene Ontology (GO) (21) enrichment analysis using R software topGO package (2.24.0) (22). Pathway enrichment analysis was performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) (21) via R software clusterProfiler package (3.0.5) (23). Terms with a P-value <0.05 were considered significantly enriched.

Primary B cell isolation. For primary B cell isolation, spleens were obtained from MRL/lpr mice. Each spleen was placed in a 70- μ m cell strainer and dissociated using a syringe plunger while rinsing with RPMI-1640 medium (cat. no. 11875093; Gibco; Thermo Fisher Scientific, Inc.) to collect cells into a tube. Following centrifugation at 300 x g for 5 min at 4°C, erythrocytes were lysed by resuspending the pellet in erythrocyte lysis buffer (cat. no. R1010; Solarbio) for 15 min at room temperature. The cells were collected at 450 x g for 10 min at 4°C and then washed, resuspended in PBS and adjusted to 1x10⁷ cells/ml. Cells were incubated with CD19 MicroBeads (cat. no. 130-121-301; Miltenyi Bio.; 10 μ l/1x10⁷ cells) for 15 min at 4°C and CD19⁺ B cells were isolated using a magnetic separation column (cat. no. 130-042-401; Miltenyi Bio.) according to the introductions.

Cell culture and treatment. Human proximal tubular human kidney-2 (HK-2) cells (cat. no. CTCC-002-0018; MeisenCTCC) were cultured in DMEM supplemented with 10% fetal bovine serum (both Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin streptomycin, under a humidified atmosphere of 5% CO₂ at 37°C. To investigate the therapeutic potential of WA, HK-2 cells were pretreated with 2.5 μ M WA for 1 h at 37°C prior to the induction of inflammation using 40 μ g/ml lipopolysaccharide (LPS) for 48 h at 37°C.

Cell transfection. To inhibit PON1 expression, HK-2 cells were transfected with either PON1-specific small interfering RNA (si-PON1; Table SI) or a PON1 overexpression plasmid (Ribobio). Cells were transfected with 1 pmol siRNA using LipofectamineTM RNAiMAX (cat. no. 13778100; Thermo Fisher Scientific, Inc.) at 37°C for 24 h before the medium was replaced with fresh complete medium and treated with 1 µg/ml LPS at 37°C for 24 h. For overexpression, the human PON1 full-length cDNA was cloned into the pcDNA3.1 plasmid vector (Ruibiotech). Cells were transfected with 0.2 µg of either the PON1 plasmid or the empty vector using LipofectamineTM 3000 (Thermo Fisher Scientific, Inc.) at 37°C for 48 h. For PON1 activation, cells were exposed to 5 µM zinc phytate (ZnPA; cat. no. 529505; Sigma-Aldrich) for 48 h at 37°C followed by 1 µg/ml LPS exposure for 24 h at 37°C. Cells were subsequently harvested for downstream protein or RNA analysis.

Cell viability assay. HK-2 cells were seeded into 96-well plates at a density of 1×10^4 cells/well. Following the respective treatments, the culture medium was aspirated and replaced with 100 µl of fresh medium containing Cell Counting Kit-8 (CCK-8) solution (cat. no. CA1210, Solarbio) in serum-free RPMI-1640. The plates were then incubated at 37°C for 1–4 h in the dark, according to the manufacturer's instructions. The absorbance at 450 nm was measured using a microplate reader.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated from kidney tissues or cultured cells using the GeneJET RNA Purification kit (cat. no. K0732; Thermo Fisher Scientific, Inc.). cDNA was synthesized from 500 ng of total RNA using the RevertAid First Strand cDNA Synthesis kit (cat. no. K1622; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. qPCR was performed in 20 µl reaction mixtures containing 2 µl of cDNA, 0.3 µM of each gene-specific primer (Table SII) and 10 µl of SYBR Green qPCR Master Mix (cat. no. K0251; Thermo Fisher Scientific, Inc.). Amplification was carried out on an ABI StepOnePlusTM Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) under the following thermocycling conditions: Initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 30 sec. All reactions were performed in triplicate. The relative levels of target genes were calculated using the $2^{-\Delta\Delta C_q}$ method (24), normalized to GAPDH.

Measurement of reactive oxygen species (ROS) levels. ROS levels were measured using a commercial kit (cat. no. EEA019; Thermo Fisher Scientific, Inc.). Briefly, following ZnPA treatments or PON1 overexpression, cells were loaded with 10 µM DCFH-DA in serum-free DMEM (Gibco; Thermo Fisher Scientific, Inc.) and incubated at 37°C for 30 min in the dark. Cells were thoroughly washed with PBS to remove excess probe and resuspended with serum-free medium for detection. The fluorescence intensity was measured using a microplate reader (excitation/emission, 488/525 nm). Results are expressed as a percentage relative to the untreated control group.

Measurement of antioxidative enzyme activity and malondialdehyde (MDA) levels. Lactate dehydrogenase (LDH)

activity was assessed using a commercial kit (cat. no. BC0625; Beijing Solarbio Science & Technology Co., Ltd.) according to the manufacturer's protocol. Following si-PON1 transfection and incubation with WA for 24 h at 37°C, the culture supernatant was mixed with the substrate solution. The mixture was incubated in the dark at room temperature for 30 min. After adding the stop solution, the absorbance was measured at 450 nm using a microplate reader. LDH activity was calculated based on the standard curve. Intracellular levels of MDA and superoxide dismutase (SOD) were assessed using commercial assay kits (cat. nos. BC6415 and BC5165, respectively; Beijing Solarbio Science & Technology Co., Ltd.) according to the manufacturer's instructions. The absorbance for each assay was measured at 532 and 600 nm for MDA or 450 nm for SOD using a microplate reader.

Western blotting. Total protein was extracted from kidney tissue or cultured cells using RIPA lysis buffer (cat. no. R0010; Beijing Solarbio) supplemented with protease and phosphatase inhibitors (cat. no. 78440; Thermo Fisher Scientific), and the protein concentration was quantified using the Bradford assay (cat. no. PC0010; Solarbio). A total of 20 µg of protein lysate were separated via 4–20% SDS-PAGE and transferred onto PVDF membranes. The membranes were blocked with 5% non-fat milk for 1 h at room temperature and incubated overnight at 4°C with anti-PON1 (1:1,000; cat. no. ab92466; Abcam), anti-TNF-α (1:1,000; cat. no. ab183218; Abcam), anti-KIM-1 (1:1,000; cat. no. ab323414; Abcam), anti-IL-1β (1:1,000; cat. no. ab283818; Abcam), anti-Bcl-2 (1:1,000; cat. no. ab182858; Abcam), anti-Bax (1:1,000; cat. no. ab32503; Abcam), anti-PPAR-α (1:1,000; cat. no. ab61182; Abcam), anti-PPARγ (1:1,000; cat. no. ab45036; Abcam) and anti-GAPDH (1:2,000; cat. no. ab8245; Abcam) as the internal reference control. After washing three times with TBST with 0.1% Tween-20, the membranes were incubated with HRP-conjugated secondary antibodies (1:5,000; cat. no. HA1001; HuaBio) for 1 h at room temperature. Protein bands were visualized using an ECL detection kit (cat. no. 32209; Thermo Fisher Scientific, Inc.). Quantification of the protein bands was performed using ImageJ software (version 1.54g).

Statistical analysis. Data are expressed as the mean ± standard error of the mean of ≥3 independent experimental repeats. Data were analyzed using one-way ANOVA followed by Tukey's post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference. All statistical analyses were performed using GraphPad Prism 8 (Dotmatics).

Results

WA is a candidate for treating SLE-associated LN. To identify potential therapeutic agents for SLE-associated LN, the present study analyzed transcriptomic profiles of peripheral immune cells from the public datasets GSE135779, GSE162577 and GSE142016. Given the key role of B cells in SLE pathogenesis (25), DEGs were identified in B lymphocytes from patients with SLE compared with those from healthy donors (adjusted $P < 0.05$). Moreover, the top 150 up- and downregulated genes, ranked by $\log_2$ fold-change, were selected as gene signatures (Table SIII) and submitted to the

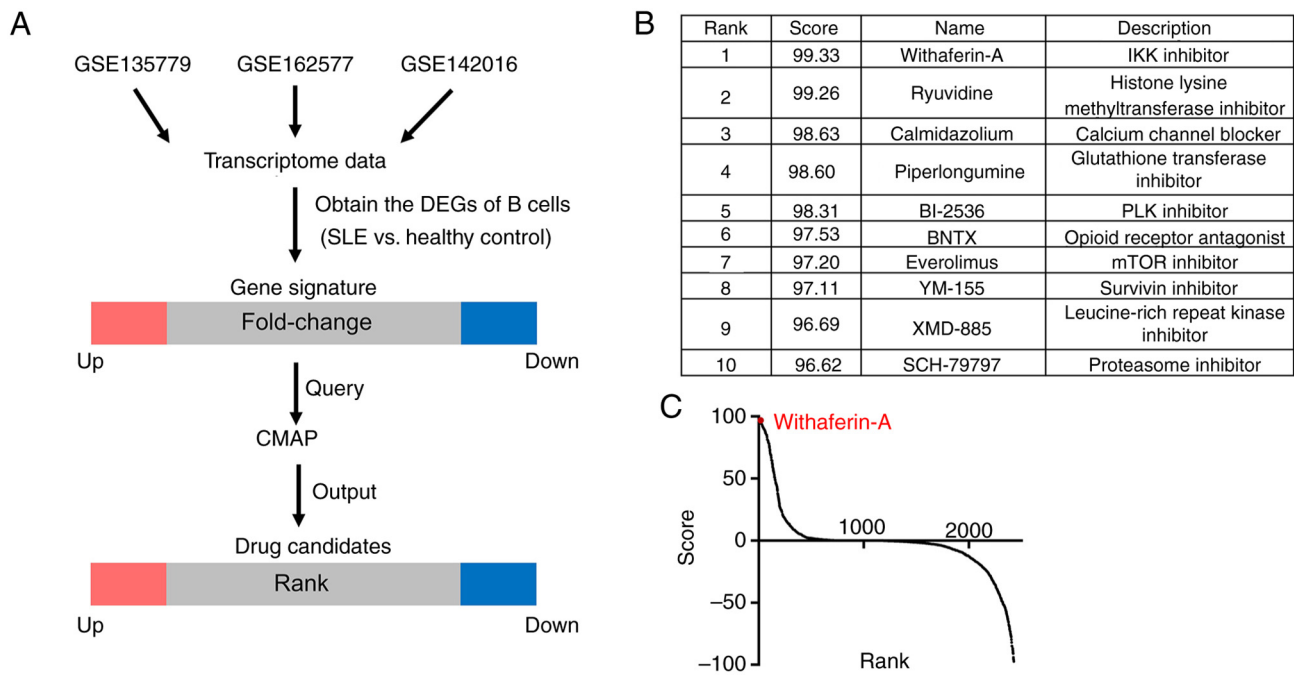


Figure 1. WA is a potential therapeutic agent for lupus nephritis. (A) CMAP query flow chart. DEGs in B cells in GSE135779, GSE162577 and GSE142016 datasets from patients with SLE and healthy donors were used to query the CMAP. (B) Top ten compounds with positive connectivity scores identified by the CMAP analysis. (C) Candidate rank according to the connectivity score. CMAP, Connectivity Map; DEG, differentially expressed gene; IKK, inhibitor of κ B kinase; PLK, polo-like kinase; SLE, systemic lupus erythematosus.

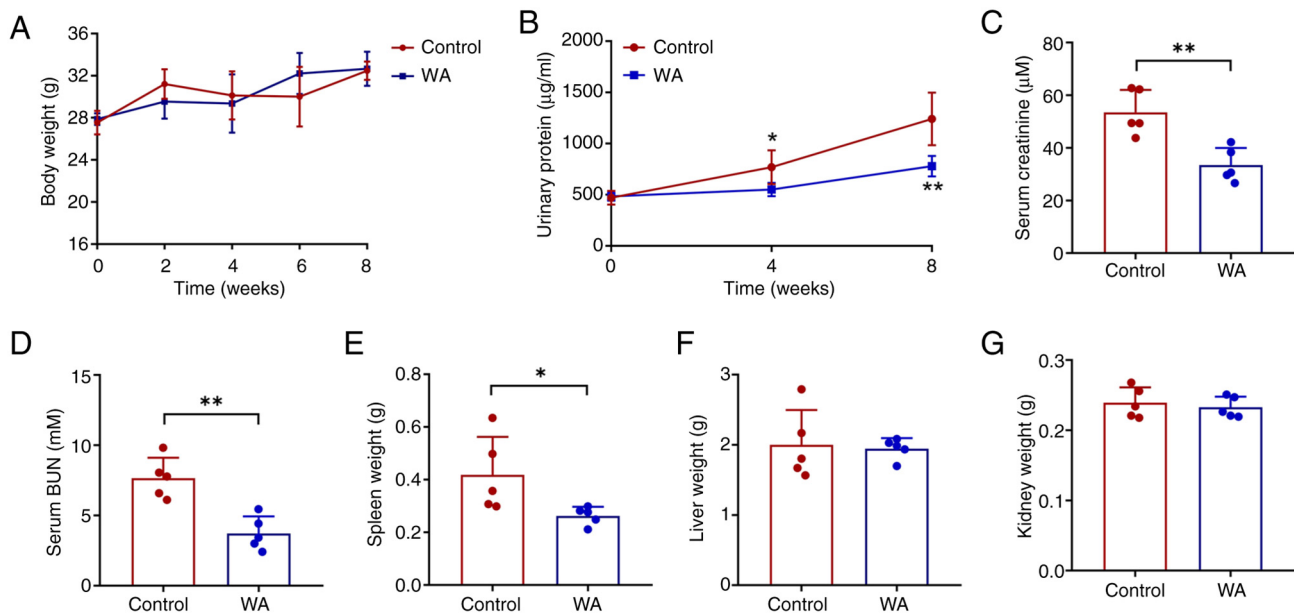


Figure 2. WA improves the renal function in lupus nephritis. (A) Body weight change in lupus mice after WA treatment. (B) Urinary protein concentrations. Serum (C) creatinine and (D) BUN levels. (E) Spleen, (F) liver and (G) kidney weight. * $P<0.05$, ** $P<0.01$ vs. control. WA, withaferin A; BUN, blood urea nitrogen.

CMAP platform to screen candidate compounds (Fig. 1A). All output compounds were ranked by CMAP score and the top 10 candidates were identified (Fig. 1B). Among these, WA ranked first (Fig. 1C), suggesting its potential as a therapeutic agent for SLE-associated LN.

WA improves renal function in experimental LN. The present study assessed the effects of WA on SLE-associated LN in

MRL/lpr mice. Following 8 week treatment, mice administered WA showed no significant difference in body weight compared with that of the control group (Fig. 2A). However, WA significantly improved renal function in lupus-prone mice, as demonstrated by significant decreases in urinary protein excretion and serum creatinine levels (Fig. 2B and C). Additionally, a significant decrease in blood urea nitrogen was observed in the WA-treated group (Fig. 2D). Notably, spleen

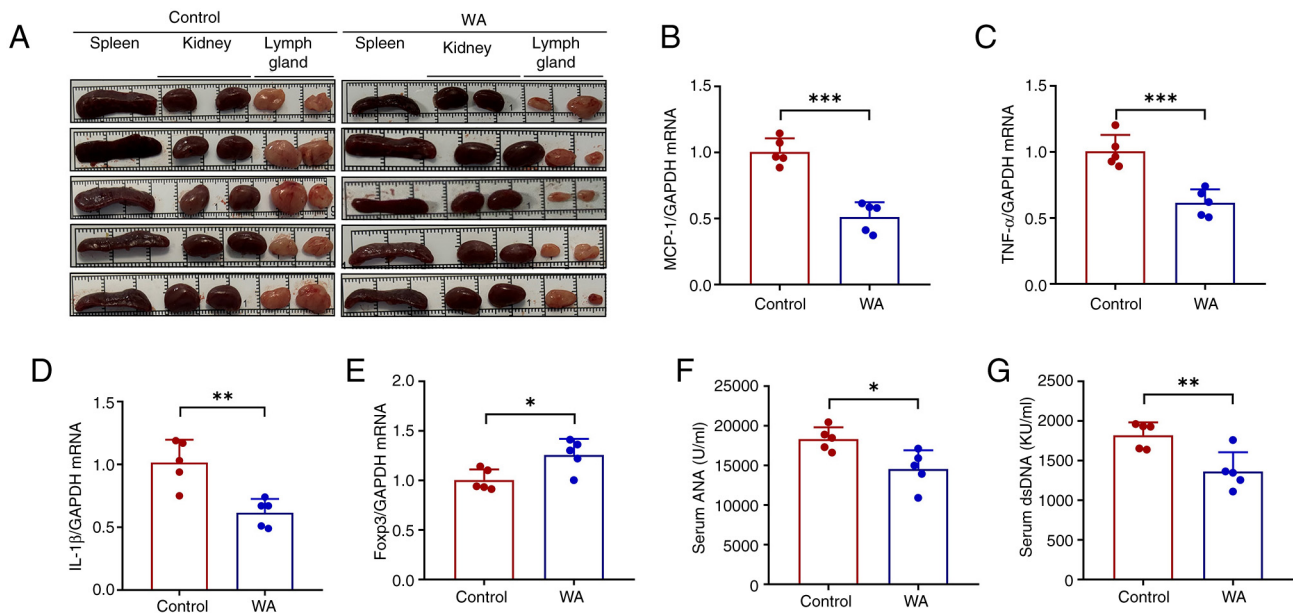


Figure 3. WA attenuates splenic immune cell dysfunction in lupus nephritis. (A) Representative spleen, lymph node and kidneys from lupus mice in the presence or absence of WA treatment. Splenic mRNA expression of (B) MCP-1, (C) TNF- α , (D) IL-1 β and (E) Foxp3. Serum (F) ANA and (G) dsDNA levels. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. WA, withaferin A; MCP, monocyte chemoattractant protein; KU, kilo unit; ANA, antinuclear antibody; ds, double-stranded.

weight was significantly lower in WA-treated mice, whereas liver and kidney weight remained comparable between the two groups (Fig. 2E-G).

WA ameliorates splenic immune cell dysfunction in LN. SLE involves splenic immune cell dysfunction that contributes to multi-organ pathology (26). SLE model mice displayed marked splenomegaly and enlarged axillary lymph nodes, both of which were attenuated by WA treatment (Fig. 3A). WA administration downregulated the splenic expression of pro-inflammatory cytokines, including monocyte chemoattractant protein (MCP)-1, TNF- α and IL-1 β (Fig. 3B-D), while upregulating the anti-inflammatory transcription factor forkhead box P3 (Fig. 3E). WA treatment significantly decreased serum levels of ANA and dsDNA autoantibodies (Fig. 3F and G). Collectively, these results demonstrated that WA ameliorates splenic immune dysfunction in murine lupus.

WA suppresses renal pathohistological changes in experimental LN. Renal pathological changes were assessed using H&E and PAS staining. Kidney sections from SLE model mice exhibited glomerular abnormality, including diffuse endothelial cell proliferation, segmental sclerosis and tubular atrophy. These pathological features were markedly attenuated by WA, consistent with the observed improvements in renal function (Fig. 4A-D). To evaluate glomerular injury, immunofluorescence staining was performed, which revealed that WA significantly decreased glomerular deposition of IgG and C3 in SLE mice (Fig. 4E-G).

WA alleviates renal inflammation in experimental LN. Immune cell-mediated sustained inflammation contributes to the pathogenesis of renal injury in lupus (27). In the present study, WA ameliorated lupus-induced renal pathology, as demonstrated by a decrease in α -SMA positive areas

(Fig. 5A and B). RT-qPCR and western blotting demonstrated that WA abrogated the upregulation of key proinflammatory mediators, including IL-1 β , TNF- α and MCP-1, in the kidney of SLE mice (Fig. 5C-F). In addition, the tubular injury was also attenuated, demonstrated by a decrease in the expression of tubular injury marker KIM-1 (Fig. 5F). Collectively, these findings indicated that WA attenuated tubular injury by suppressing the intrarenal inflammatory response.

Pon1 is a mediator of alleviated innate immune responses induced by WA. The present study performed genome-wide transcriptomic sequencing on renal tissue from SLE mice with or without WA treatment to elucidate the molecular basis of its immunosuppressive effects. Notably, WA markedly reshaped the renal transcriptome (Fig. 6A), enhancing the expression of lipid metabolism genes [perilipin (PLIN)4, fatty acid-binding protein 4 (FABP4) and PLIN5] and suppressing genes associated with immunity [lymphocyte antigen 6 complex locus D, immunoglobulin heavy variable 5-9, C-X-C motif chemokine ligand (CXCL)10 and CXCL13], transcription (STAT4 and transcription factor 7) and apoptosis (growth arrest and DNA damage-inducible γ and serine/threonine kinase 17B; data not shown). GO and KEGG analyses demonstrated that WA activated lipid-centric and metabolic pathways while inhibiting pro-inflammatory signaling (Fig. 6B and C). Moreover, from the 161 DEGs identified by volcano plot (18 up- and 143 down-regulated), Pon1 emerged as a lead candidate as the second most significantly upregulated transcript in the WA-treated group (Fig. 6D and E). Among the top upregulated genes identified by RNA-seq, Pon1 was selected for further functional validation due to its roles in alleviating oxidative stress and systemic inflammation (28-31), both of which are hallmark features of SLE-induced renal damage. Therefore, it was hypothesized that induction of Pon1 is a critical mechanism through which WA alleviates SLE pathology.

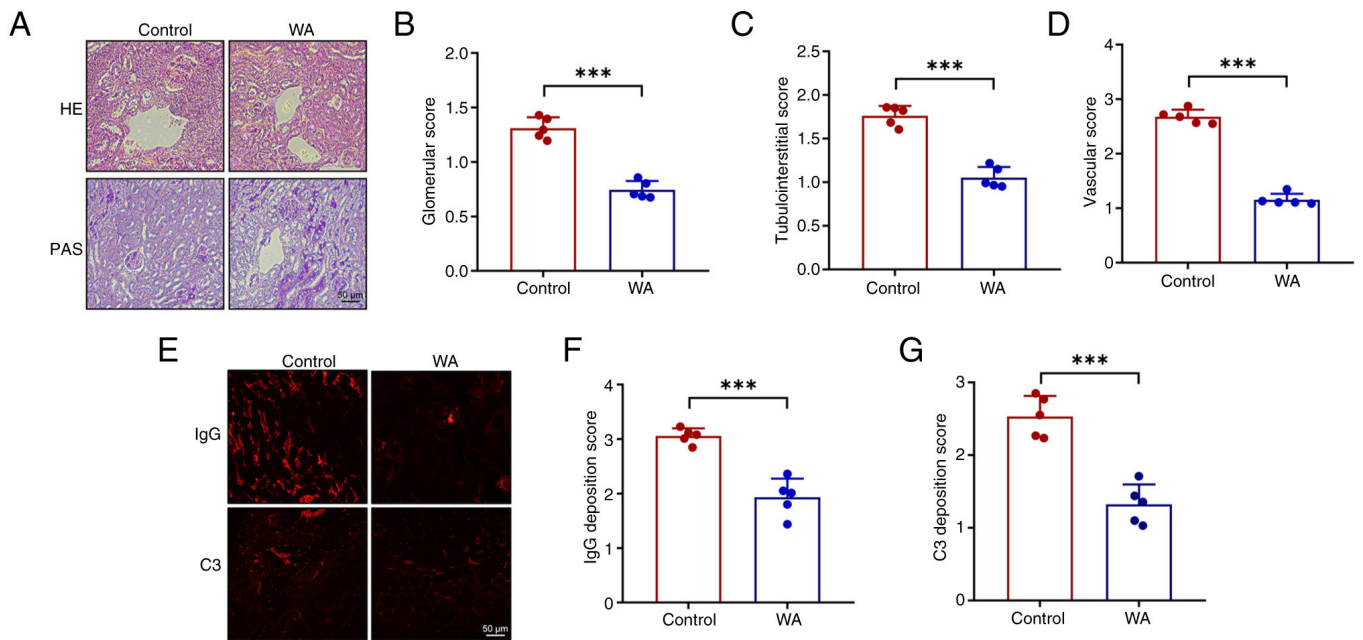


Figure 4. WA suppresses renal pathohistological changes in lupus nephritis. (A) Representative histological images of the kidney from lupus mice. Scale bar, 50 μ m. Quantitative analysis of (B) glomerular, (C) tubulointerstitial and (D) vascular scores. (E) Representative glomerular expression of (F) IgG and (G) C3 in the kidneys from lupus mice. Scale bar, 10 μ m. ***P<0.001. WA, withaferin A; HE, hematoxylin eosin; PAS, periodic acid Schiff.

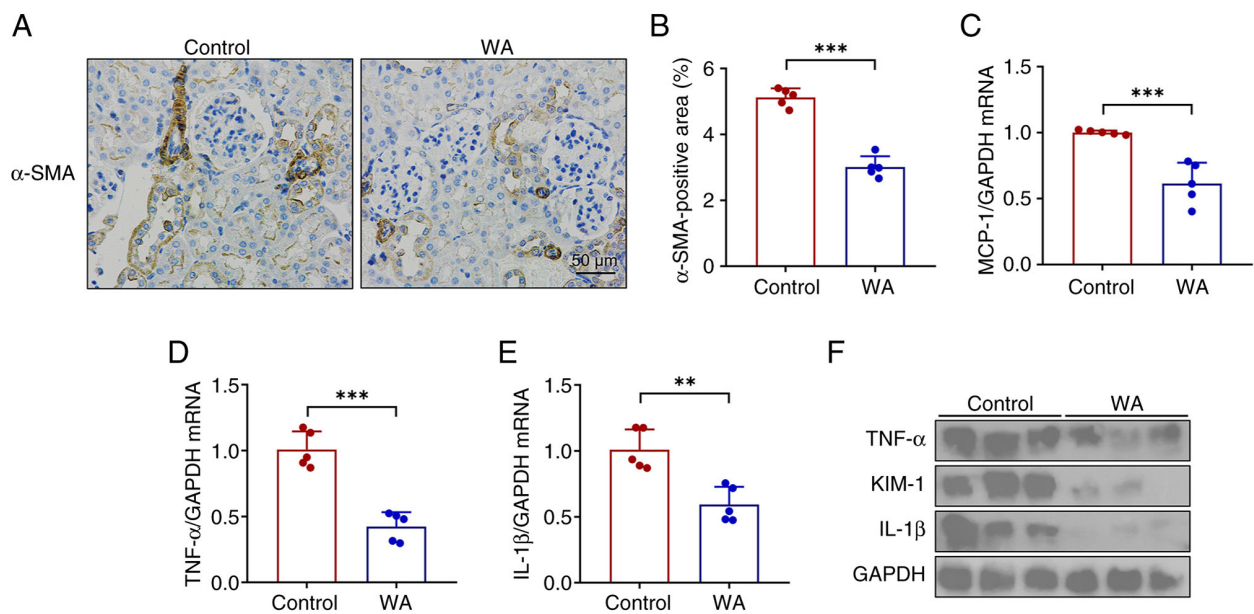


Figure 5. WA alleviates renal inflammation in lupus nephritis. (A) Representative immunohistochemical staining of (B) α -SMA expression in the kidney of lupus mice after WA treatment. Scale bar, 50 μ m. Renal mRNA levels of (C) MCP-1, (D) TNF- α and (E) IL-1 β . (F) Western blotting analysis of renal MCP-1, TNF- α , IL-1 β and KIM-1 expression. **P<0.01, ***P<0.001. WA, withaferin A; SMA, smooth muscle actin; MCP, monocyte chemoattractant protein; KIM, kidney injury molecule.

Inhibition of Pon1 reverses the effects induced by WA in HK-2 cells under inflammatory conditions. To assess the role of Pon1, the present study established an *in vitro* model of LN by stimulating HK-2 cells with LPS. WA significantly restored the impaired cell viability induced by LPS (Fig. 7A) and suppressed the upregulation of proinflammatory cytokines (IL-1 β , TNF- α and MCP-1) at both the mRNA and protein levels (Fig. 7B-E). Notably, WA also upregulated Pon1 expression (Fig. 7F).

To determine whether Pon1 is essential for the effects of WA, the present study performed loss-of-function experiments using siRNA-mediated knockdown of Pon1 (Fig. S1) and abrogated the protective effects of WA, as demonstrated by a reversal of the improved cell viability (Fig. 7A) and increased proinflammatory cytokine expression (Fig. 7B-E). Given previous findings that Pon1 mitigates cellular stress by activating PPAR γ signaling (32) and the transcriptomic

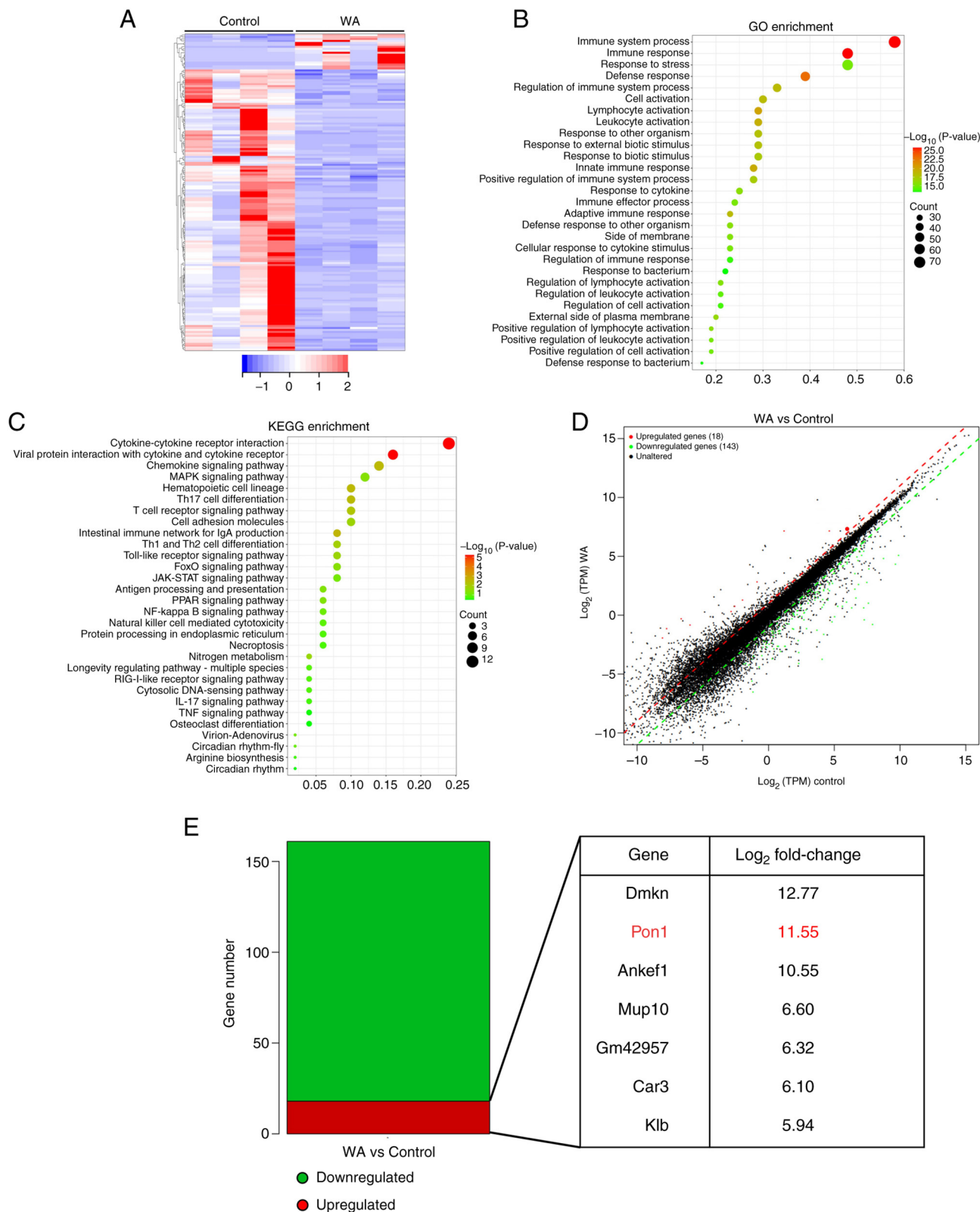


Figure 6. RNA sequencing analysis of kidney in SLE mice after WA treatment. (A) Hierarchical clustered heatmap of gene expression profiles for WA or vehicle-treated SLE mice. (B) GO enrichment analysis of DEGs. (C) KEGG pathway analysis of DEGs. (D) Volcano plot of gene expression profiles. (E) Number of DEGs ($q < 0.05$). The top seven genes are presented. SLE, systemic lupus erythematosus; WA, withaferin A; GO, gene ontology; DEG, differentially expressed genes; KEGG, Kyoto encyclopedia of genes and genomes; TPM, transcripts per kilobase; Dmkn, dermokine; Pon, paraoxonase; Ankef, ankyrin repeat and EF-hand domain containing; Mup, major urinary protein; Gm, predicted gene; Car, carbonic anhydrase; Klb, klotho β .

data in the present study indicating WA activates the PPAR pathway *in vivo*, this pathway was investigated. WA enhanced the expression of PPAR α and PPAR γ in LPS-treated HK-2

cells (Fig. 7G). This activation was associated with attenuated oxidative stress, demonstrated by decreased levels of LDH and MDA and an increase in SOD activity (Fig. 7H-J). WA

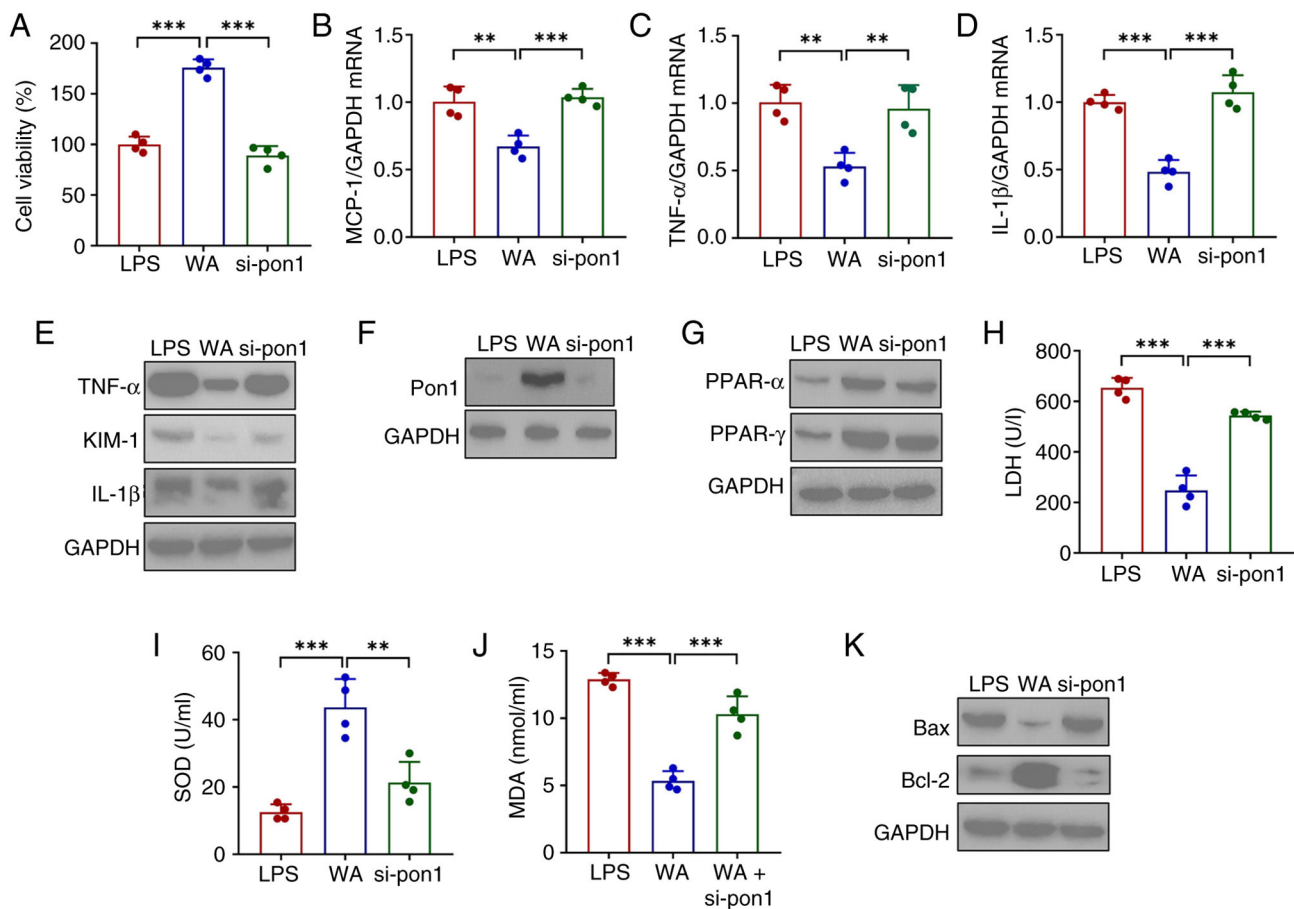


Figure 7. WA alleviates inflammation and eliminates reactive oxygen species in HK-2 cells by regulating Pon1/PPAR signaling under inflammatory conditions. (A) Viability of HK-2 cells after WA treatment in the presence or absence of si-Pon1 transfection. All cells were pretreated with LPS. mRNA expression of (B) MCP-1, (C) TNF- α and (D) IL-1 β and (E) western blotting analysis of the expression of MCP-1, TNF- α , IL-1 β and KIM-1 in HK-2 cells. (F) Pon1 expression levels. (G) Protein expression of PPAR- α and PPAR- γ . (H) LDH and (I) SOD activities. (J) MDA levels in HK-2 cells. (K) Expression of Bax and Bcl-2. **P<0.01, ***P<0.001. WA, withaferin A; Pon, paraoxonase; si, small interfering; LPS, lipopolysaccharide; MCP, monocyte chemoattractant protein; LDH, lactate dehydrogenase; SOD, superoxide dismutase; MDA, malondialdehyde.

modulated the expression of apoptosis-related proteins, as indicated by the downregulated expression of Bax and Bcl-2 (Fig. 7K). Notably, these beneficial regulatory effects of WA were nullified by Pon1 knockdown (Fig. 7G-K).

WA led to a decrease in supernatant IgG (Fig. 8A) and diminished expression of the B cell surface markers CD19, CD20, CD21 and CD22 (Fig. 8B-E), suggesting suppressed B cell differentiation and proliferation. Conversely, an increase in the apoptosis-associated marker CD24 was observed (Fig. 8F), indicating promotion of B cell apoptosis.

Pon1 activator alleviates innate immune responses in HK-2 cells. As Pon1 demonstrated a role in WA-induced effects, the present study assessed whether Pon1 activator showed similar effects induced by WA. ZnPA significantly enhanced the expression of Pon1 (Fig. 9A) in HK-2 cells. ZnPA increased the viability of HK-2 cells treated with LPS (Fig. 9B), which was accompanied by decreased expression of the proinflammatory cytokines IL-1 β , TNF- α and MCP-1 (Fig. 9C). Notably, PPAR signaling, oxidative stress and apoptosis induced by LPS were markedly attenuated in HK-2 cells following ZnPA administration (Fig. 9D-F). Pon1 was overexpressed in HK-2 cells to evaluate whether higher levels of Pon1 led to similar

kidney protection (Fig. 10A). Pon1 overexpression resulted in increased cell viability (Fig. 10B) and decreased inflammation (Fig. 10C-E) and ROS levels (Fig. 10F). Relative mRNA expression of Pon1 was compared between healthy donors and patients with LN in the public dataset GSE121239. Pon1 expression was significantly lower in the patients with LN than in healthy donors (Fig. S2), supporting the hypothesis that augmenting Pon1 expression represents a promising therapeutic strategy for LN.

Discussion

SLE is a chronic autoimmune disorder marked by diverse clinical manifestations that impact multiple organ systems. Therapeutic strategies for SLE primarily aim to suppress the immune response. However, the complexity of SLE pathophysiology highlights the need to elucidate the underlying molecular mechanisms and key signaling pathways involved in disease progression, as well as to develop novel, targeted therapies with enhanced efficacy and safety profiles.

The present study identified WA as a promising therapeutic agent capable of alleviating SLE symptoms both *in vivo* and *in vitro*. Mechanistically, WA upregulated Pon1 expression

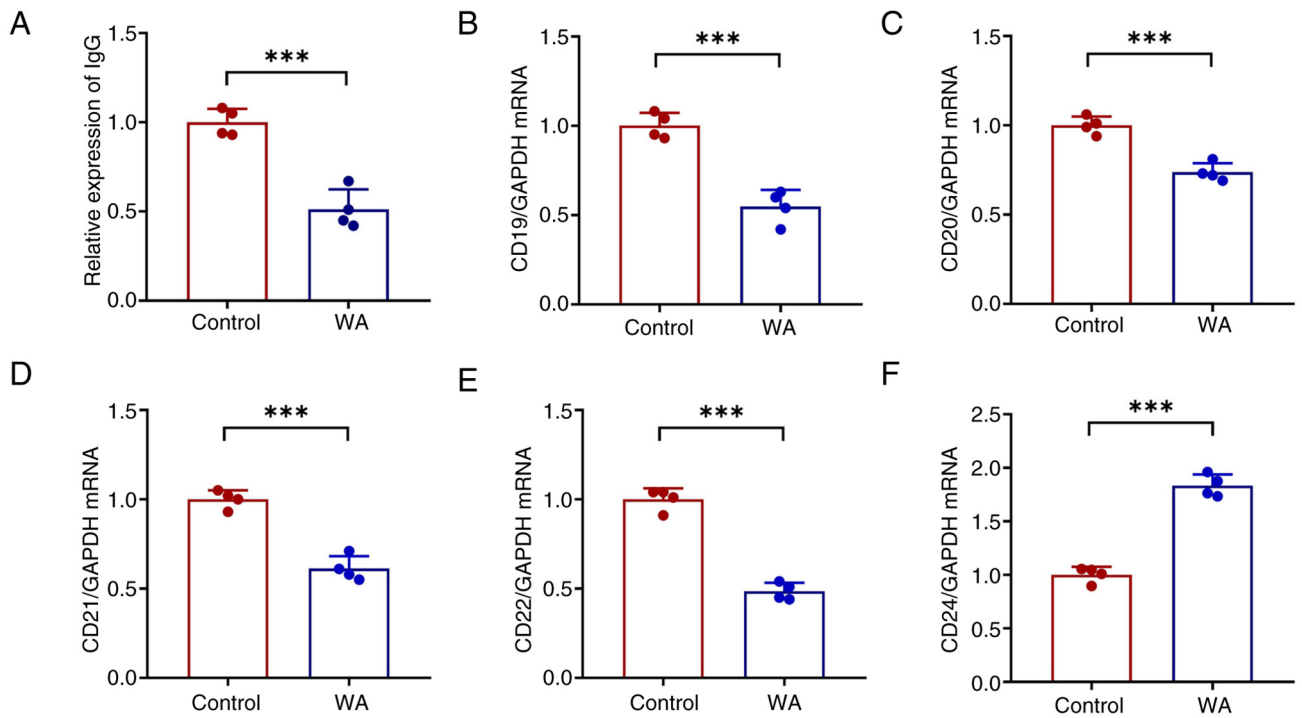


Figure 8. WA suppresses activation of B cells from systemic lupus erythematosus mice. (A) IgG levels in B cell culture medium. mRNA levels of genes related to B cell differentiation, including (B) CD19, (C) CD20, (D) CD21 and (E) CD22. (F) mRNA levels of CD24. ***P<0.001. WA, withaferin A.

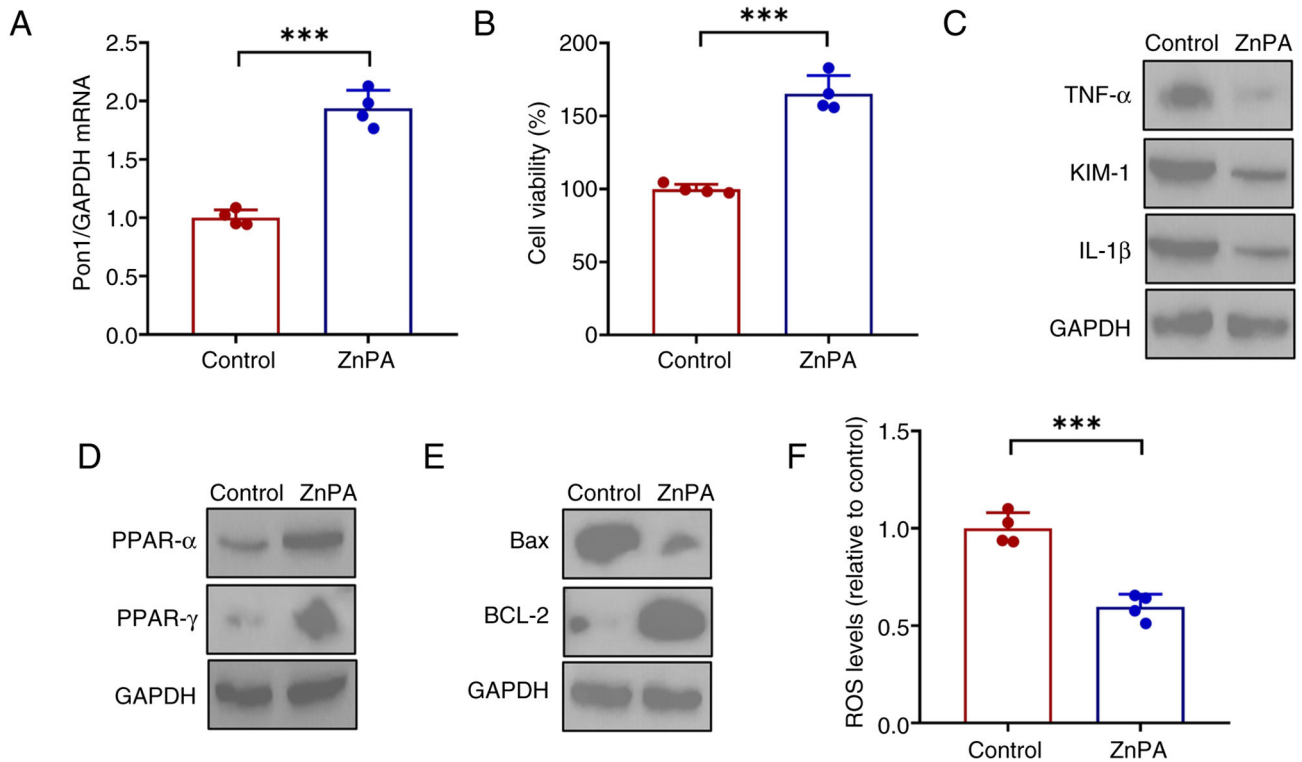


Figure 9. Pon1 activation improves the inflammation and eliminates ROS in HK-2 cells. (A) Pon1 expression in HK-2 cells following ZnPA treatment. (B) Viability of HK-2 cells following ZnPA treatment. All cells were pretreated with lipopolysaccharide. (C) Western blotting analysis of MCP-1, TNF- α , IL-1 β and KIM-1 expression. (D) Protein expression of PPAR- α and PPAR- γ . (E) Expression of Bax and BCL-2. (F) ROS levels. ***P<0.001. ZnPA, (Z)-2,3-bis (4-nitrophenyl)-acrylonitrile; Pon, paraoxonase; MCP, monocyte chemoattractant protein; KIM, kidney injury molecule; ROS, reactive oxygen species.

in the kidney and decreased splenic and renal inflammation, oxidative stress and apoptosis through activation of the PPAR signaling pathway. ZnPA, a Pon1 activator, countered

LPS-induced cytotoxicity, suppressed excessive immune activation and diminished oxidative stress and the expression of apoptosis-associated proteins in HK-2 cells, underscoring the

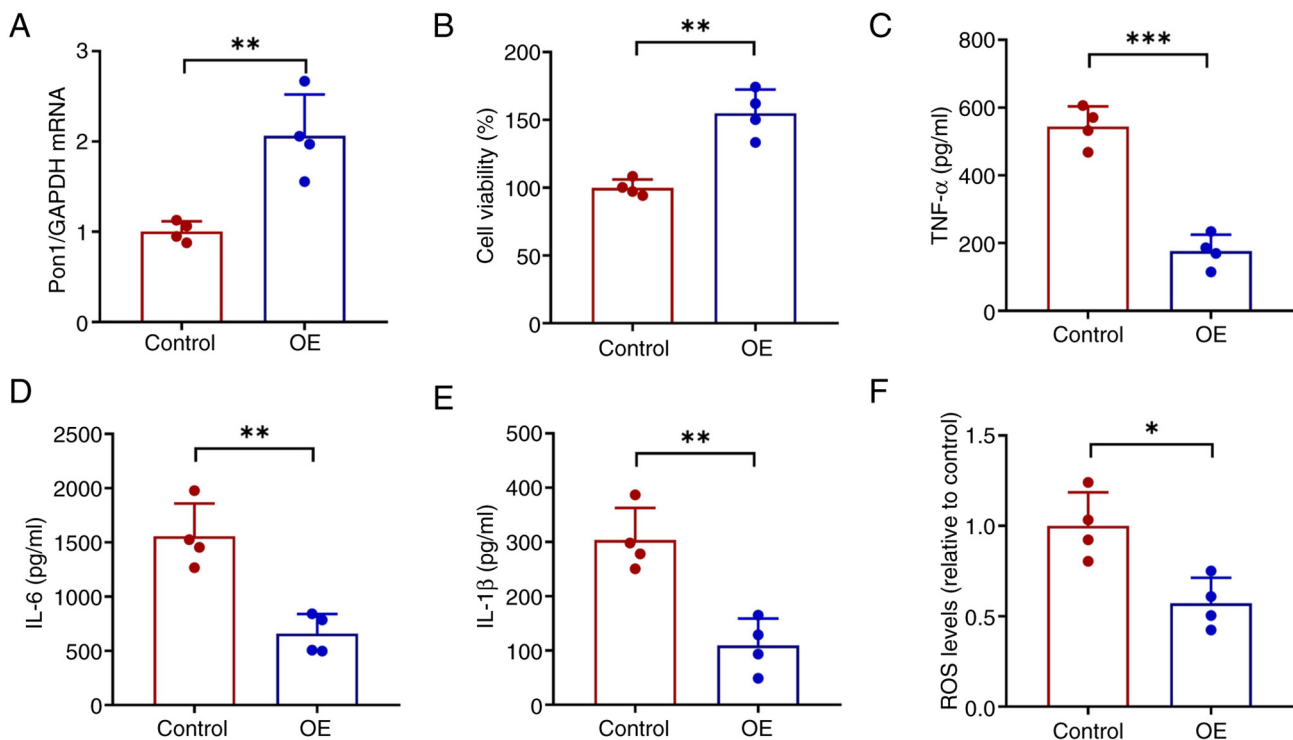


Figure 10. Pon1 overexpression attenuates inflammation and eliminates ROS in HK-2 cells. (A) Pon1 expression in HK-2 cells following plasmid transfection. (B) Viability of HK-2 cells following plasmid transfection. All cells were pretreated with lipopolysaccharide. ELISA of (C) TNF- α , (D) IL-6 and (E) IL-1 β . (F) ROS levels. *P<0.05, **P<0.01, ***P<0.001. OE, overexpression; Pon, paraoxonase; ROS, reactive oxygen species.

potential of Pon1-targeted interventions for the treatment of SLE.

WA, a steroidal lactone derived from the plant *W. somnifera*, is utilized as a natural compound in traditional medicine due to its broad-spectrum efficacy in several disorders, such as Parkinson's disease (33) and obesity (34). Additionally, WA is a potent antioxidant (35), anti-inflammatory (36), anti-angiogenic (37), anti-epithelial-mesenchymal transition (38) and immunomodulatory agent (39). WA can mitigate oxidative stress-induced apoptotic death in cardiomyocytes (40) and protect against free fatty acid-induced inflammatory responses and oxidative stress by inhibiting IL-6 production and suppressing NF- κ B signaling in endothelial cells (41). Kanak *et al* (42) reported that administering WA, an NF- κ B inhibitor, inhibits endoplasmic reticulum stress and inflammasome activity to moderate chronic pancreatitis in a mouse model. Moreover, the present study identified WA as a potential therapeutic agent for SLE through the CMAP approach. WA significantly improved renal function in a murine model of SLE. Furthermore, WA ameliorated splenic immune cell dysregulation, attenuated renal pathohistological abnormality and decreased renal inflammation. Integrated RNA-seq analysis and siRNA transfection revealed that Pon1 serves a pivotal role in mediating the protective effects of WA by mitigating oxidative stress and apoptosis. The present findings also highlight the multi-organ protective profile of WA, showing no obvious changes in body weight and liver weight. Pires *et al* (43) reported clinical adverse effects in patients with advanced-stage high-grade osteosarcoma treated with WA, including fever, fatigue, gastrointestinal reaction and elevated liver enzymes. These may be disease-related complications

or sequelae of the advanced clinical stage rather than direct toxicities of WA. Advanced high-grade osteosarcoma is typically associated with systemic inflammatory responses and hypoalbuminemia, which predispose patients to fever and edema, both of which are common clinical hallmarks of disease progression. Furthermore, these symptoms were also observed in the control group. The incidence of these events did not exhibit a clear dose-response association, further suggesting they were independent of WA administration (43). Consequently, further research is warranted to delineate the clinical effects attributable to WA treatment. The aforementioned study found WA to be well-tolerated by patients at doses up to 216 mg/day (43). The dose in the present study (2 mg/kg/day, equivalent to 9.8 mg/day for a 60 kg human) was substantially lower. Therefore, it was unlikely to cause observable toxicity and was safe for the regular use. Nevertheless, the lack of comprehensive, longitudinal pharmacokinetic profiles and human preclinical data remains a limitation of the present study. It is necessary to determine the association between systemic exposure of WA and clinical complications such as fatigue or hepatotoxicity.

As a circulating calcium-dependent esterase and lactonase, Pon1 is primarily synthesized in the liver, kidney and intestine (44), and is predominantly associated with the high-density lipoprotein (HDL) fraction in circulation (45). This contributes to the antioxidant and antiatherogenic properties of HDL (44,46). HDL is a key mediator of reverse cholesterol transport (47,48), facilitating the removal of excess cholesterol from peripheral tissue. Accumulating evidence has revealed the functional complexity of HDL in several pathological conditions, including infection, neoplasms and

autoimmune diseases (49-52). The pleiotropic functions of HDL include anti-inflammatory, antioxidant, antithrombotic and antiapoptotic effects and the promotion of nitric oxide synthesis (49). In SLE, numerous patients exhibit elevated levels of very-low-density lipoprotein and low-density lipoprotein, along with decreased HDL levels, a profile commonly referred to as the lupus lipoprotein pattern (50-52). Low levels of HDL-cholesterol represent one of the most prevalent dyslipidemia markers in SLE, including among pediatric populations. Notably, additional HDL-associated abnormalities have been reported, such as diminished Pon1 activity (53), suggesting that modulation of HDL function via Pon1 regulation may serve as an effective therapeutic strategy for SLE. In the present study, Pon1 was identified as a potential molecular target of WA in the treatment of SLE. Upregulated Pon1 expression in both kidney tissue and HK-2 cells following WA administration was demonstrated and siRNA-mediated knockdown and pharmacological activation assays suggested that Pon1 mediates the beneficial effects of WA in SLE via the PPAR signaling pathway. These findings support Pon1 as a promising therapeutic target for SLE intervention. RNA-seq revealed activation of the PPAR signaling pathway and upregulation of key lipid metabolism-associated genes, including PLIN4, FABP4 and PLIN5. PPAR signaling transcriptionally regulates energy metabolism and inflammatory responses. Patients with LN typically exhibit a distinct lupus lipoprotein pattern, characterized by systemic and intrarenal lipid dysregulation (54). The present study indicated that WA not only enhanced the expression of PPAR α and PPAR γ in HK-2 cells but also promoted the expression of proteins essential for lipid droplet formation and fatty acid transport. These metabolic modulations may contribute to the reinforcement of energy homeostasis within renal proximal tubular cells by mitigating ectopic lipid deposition and its associated lipotoxicity, thereby alleviating renal injury. Furthermore, Pon1 is key in maintaining lipid and redox stability. Pon1 activation can mitigate cell stress by triggering PPAR γ signaling (32). The present study further demonstrated this regulatory axis, as the beneficial effects of WA on PPAR protein levels were abolished upon Pon1 knockdown. The activation of the Pon1/PPAR pathway may be key for the suppression of ROS. Mechanistically, the PPAR pathway inhibits pro-inflammatory cascades, such as NF- κ B, while bolstering antioxidant defenses, effectively breaking the cycle of intrarenal inflammation and oxidative damage (55).

B cell dysfunction serves a key role in the pathogenesis of SLE (56). Under physiological conditions, B cell activation is regulated by several factors, including ROS (57). ROS contribute to B cell maturation (58-60). For example, hydrogen peroxide enhances the DNA-binding activity of paired box 5, and exposure of B cells to H₂O₂ induces rapid nuclear translocation of the cytoplasmic redox factor apurinic/aprimidinic endonuclease 1/redox effector factor-1, thereby promoting early B cell development and maturation (58,59). In addition, ROS regulate B cell activation through phosphorylation of spleen tyrosine kinase and other membrane-proximal B cell receptor signaling effectors (60). ROS levels are elevated in activated B cells, and oxidative processes are key for B cell differentiation. ROS production increases progressively during B cell activation and differentiation (57). The RNA-seq

analysis in the present study demonstrated downregulation of several Ig and IL genes, including Ig heavy variable 8-12, immunoglobulin heavy constant ϵ and IL-21 (data not shown), suggesting suppressed B cell activation. As WA decreased ROS levels, it was hypothesized that this reduction contributes to inhibition of B cell activation in SLE mice. WA acts on HK-2 cells to decrease ROS generation via the Pon1/PPAR signaling pathway, thereby inhibiting B cell activation and ameliorating SLE symptoms. Furthermore, RNA-seq revealed upregulation of several lipid metabolism-associated factors in WA-treated mice such as *plin5*, *pnpla3* and *plin4*. These changes may also be associated with PPAR signaling as this pathway suppresses ROS production (61), although the underlying mechanisms require further investigation.

The present study has certain limitations. First, although Pon1 was identified as a potential therapeutic target for SLE, additional evidence is needed to establish its functional role in SLE models. The present study did not assess whether Pon1 overexpression protects against LN-induced kidney injury. Second, the present study did not determine whether WA directly targets B cells to modulate their activation and correct B cell dysfunction in SLE. The present findings indicated that WA enhances Pon1 expression and suppresses ROS production through PPAR signaling, leading to inhibition of B cell activation and alleviation of SLE-associated LN in mice.

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Availability of data and materials

The datasets generated and/or analyzed during the current study are available in the Sequence Read Archive dataset under accession no. PRJNA1393487 or at the following URL: ncbi.nlm.nih.gov/bioproject/PRJNA1393487.

Authors' contributions

MX and XM conceived and designed the study. MX and JF performed the language editing, analyzed data and constructed figures. MX performed bioinformatics analysis. All authors have read and approved the final manuscript. MX, JF and XM confirm the authenticity of all the raw data.

Ethics approval and consent to participate

All animal experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Experimental Ethical Inspection of Gansu University of Chinese Medicine (approval no. SY2024-257; Lanzhou, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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