Therapeutic potential of DCB-SLE1, an extract of a mixture of Chinese medicinal herbs, for severe lupus nephritis

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LUPUS NEPHRITIS, ONE OF THE MOST SEVERE complications of systemic lupus erythematosus, is associated with significant morbidity and mortality (4, 10, 42). Accelerated severe lupus nephritis (ASLN) is characterized by acute onset of renal function impairment and the presence of cellular crescents, neutrophil infiltration, fibrinoid necrosis, extensive interstitial inflammation, and tubular atrophy in the kidney (5, 24, 34, 48, 54). The critical role of abnormal T cell and B cell function in the innate and adaptive immune responses in lupus nephritis is well established (3, 17, 62). In addition to the involvement of primary mediators, including autoantibody production and immune complex deposition (4, 13, 57), cytokines are highly implicated in the development and progression of lupus nephritis, especially Th1 and Th17 cytokines (15, 56, 60, 61, 68). These cytokines are involved not only in the immune dysregulation but also in the local inflammatory response, in which cellular immune effectors, such as T cells, macrophages, and neutrophils, cause renal injury in most cases of ASLN (22, 46–48, 56, 65). Importantly, impaired natural killer (NK) cell cytotoxicity has been shown to promote the development of renal lesions in lupus nephritis (20, 25, 45).

Current therapies for severe forms of lupus nephritis are various combinations of corticosteroids with other cytokitic agents or immunomodulators, such as cyclophosphamide, aza-thioprine, or cyclosporin (6, 41, 69), but many of these have various side effects (16, 28). The immunomodulatory activity of various traditional Chinese medicinal herbs is now widely acknowledged (35). DCB-SLE1, developed by the Development Center for Biotechnology (Taipei, Taiwan, ROC) and approved as an investigational new drug (IND; approval no. 0960305986) by the Department of Health, Taiwan, is an extract of a mixture of four traditional Chinese medicinal herbs, Atractylodis macrocephalae Rhizoma, Eucommiae cortex, Lonicerae caulis, and Hedyotidis diffusae Herba, which have been used empirically for the treatment of proteinuria in Taiwan, and is currently being investigated in a double-blind, randomized, parallel, placebo-controlled phase I/II clinical trial to evaluate the safety and efficacy in systemic lupus erythematosus patients (clinical protocol no. DCBSLE1A050214). A. macrocephalae Rhizoma, a genus of Compositae, is used as a diuretic agent in patients and has been reported to increase the effectiveness of vaccination against foot-and-mouth disease in mice (37). E. cortex and L. caulis have been used as anti-inflammatory agents to improve the symptoms of liver inflammation. Recent research has shown that E. cortex contains geniposidic acid, geniposide, and aucubin, all of which have antiosteoporosis effects (21). L. caulis is used as an anti-inflammatory agent for relief of pain and has been used to increase the phagocytic activity of neutrophils (26). H. diffusae Herba is traditionally used as an anti-inflammatory agent to reduce the generation of abscesses, toxic sores, ulcerations, and swellings and minimize the effects of snakebite. H. diffusae has also been reported to contain high levels of lantanum and has been used to treat cardiovascular disorders (66).

In the present study, we examined whether DCB-SLE1 could ameliorate the symptoms and progression in an ASLN model in New Zealand Black/White (NZB/W) F1 mice (54) and the underlying mechanisms. DCB-SLE1 administration mitigated the evolution of ASLN by reducing glomerular...
proliferation, crescent formation, neutrophil infiltration, and interstitial inflammation. The mechanism involved inhibition of B cell activation and reduction of autoantibody production, inhibition of T cell activation/proliferation and NK cell cytotoxicity, prevention of inflammation by blocking systemic inflammatory cytokine expression and intrarenal NF-κB activation, and protection against lymphoid and renal apoptosis.

MATERIALS AND METHODS

DCB-SLE1 Preparation and Optimal Dose Selection

Briefly, extraction was performed by soaking a 1,500-g mixture of equal amounts of each herb in 15 liters of double-distilled water for 1 h, then boiling the suspension at 98°C for 35 min and filtering it on a no. 400 mesh filter. Extraction was performed twice, and the two filtrates were combined and evaporated to a powder (DCB-SLE1).

Based on the guidance of IND regulations in the Republic of China Taiwan Food and Drug Administration, a 24-wk oral toxicity study in BALB/c mice was performed for evaluation of the safety of DCB-SLE1 that was used in the present study. The results showed that no abnormality in appearance and in body weight and no observable pathological changes were found in the major organs of any BALB/c mice between any the study groups. Besides, in a 13-wk subchronic oral toxicity study, DCB-SLE1 showed that no ocular finding was attributed to the administration of DCB-SLE1 in Sprague-Dawley rats, and no significant difference in all hematology parameters between DCB-SLE1-treated rats and untreated normal control rats. All animals survived through the entire study period, and no difference was shown in the change in body weight at weekly intervals among DCB-SLE1 and untreated normal control groups during the 13-wk dosing period. In our preliminary study, after three consecutive 6-wk treatment courses (different dose groups) with a 2-wk interval, the dose of 12.5 g/kg body wt of DCB-SLE1 can ameliorate nephritis, including proteinuria, hematuria, and renal function, and mortality in a spontaneously occurring lupus nephritis model in NZB/W F1 mice (not administered with LPS). We therefore used this dose in the experiments.

Establishment of the ASLN Model and Experimental Protocol

The LPS-induced ASLN mouse model was established in 8-wk-old female NZB/W F1 mice by twice weekly intraperitoneal injection of LPS (0.6 mg/kg body wt, Sigma, St. Louis, MO) as described previously (54). Two days after the first injection of LPS, the mice were divided into 2 groups of 12 mice each and were given either DCB-SLE1 or vehicle (normal saline) daily via oral gavage until euthanasia. Age-matched NZB/W F1 mice injected with normal saline instead of LPS were used as the normal controls as described previously (54). Six mice in each group were euthanized at week 1 or week 5 after disease induction. The tissue specimens of spleen, renal cortical tissue, blood, and urine were collected at the indicated times and stored appropriately before analysis. All animal experiments were conducted with the ethical approval of the Institutional Animal Care and Use Committee of The National Defense Medical Center, Taiwan according to the ethical rules in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Clinical and Pathological Evaluation

The urine samples were collected in metabolic cages weekly; urine protein was measured as described previously (31), and urine blood was measured using a Urine Test Strip (Siemens, Tokyo, Japan). The serum levels of blood urea nitrogen (BUN) and creatinine were determined at the indicated time as described previously (31). The examination of renal pathology and scoring were performed in a blinded fashion by a pathologist, and the severity of renal lesions was scored as described previously (31). The percentage of glomeruli showing proliferation, neutrophil infiltration, crescent formation, fibrinoid necrosis, and periglomerular inflammation was calculated from 50 randomly sampled glomeruli.

Immune Complex Detection, Immunohistochemistry, and Detection of Apoptosis

For immune complex detection, frozen renal tissues were prepared as described previously (32) and then stained with FITC-conjugated goat antibodies against mouse IgG, IgM, or C3 (Cappel). Fifty glomeruli were examined on each slide and assigned values of staining intensity from 0 to 3. The total intensity score was calculated for each specimen using the equation as follows: total intensity score = (% of glomeruli with a negative intensity score × 0) + (% of glomeruli with a trace intensity score × 0.5) + (% of glomeruli with a 1+ intensity score × 1) + (% of glomeruli with a 2+ intensity score × 2) + (% of glomeruli with a 3+ intensity score × 3); the values therefore ranged from 0 to a maximum of 300 (32).

For immunohistochemistry (IHC), formalin-fixed and paraffin-embedded renal sections were prepared as described previously (32), and primary antibodies against mouse CD3 (pan-T cell; Serotec), CD4 (T helper cell; BioLegend), F4/80 (monocytes/macrophages; Serotec), CD11b (macrophages/neutrophils; BD Biosciences), monocyte chemotactic protein 1 (MCP-1; Santa Cruz Biotechnology, Santa Cruz, CA), IL-6 (R&D Systems, Minneapolis, MN), or NF-κB p65 (Cell Signaling Technology, Danvers, MA) were used, respectively, and then secondary biotinylated antibodies (Dako, Glostrup, Denmark) and avidin-biotin-peroxidase complex (Dako). For studying CD8, frozen sections were fixed in acetone for 5 min and stained with primary antibodies against mouse CD8 (T cytotoxic cell, BD Biosciences). The Alexa Fluor 594-conjugated secondary antibody (Invitrogen, Carlsbad, CA) was then applied to the sections. The isotype-matched IgG from the same species of the primary antibodies was used as an antibody control for IHC staining. Semiquantitative evaluation of staining was performed as described previously (32).

For the detection of apoptosis, a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed. Paraffin-embedded sections were stained using an ApopTag Plus Peroxidase in Situ Apoptosis Detection kit (Chemicon) according to the manufacturer’s instructions, and the number of apoptotic cells in the spleen or cortical renal area (including glomerular and periglomerular area) was counted as the number of TUNEL-positive cells per field and per glomerular cross section, respectively, as described previously (32).

Analyses of T/B Cell Activation, T Cell Proliferation, and NK Cell Activity

The mice splenocytes were isolated as described previously (32) for T or B cell activation, T cell proliferation, or NK cell activity assays. For assessment of T or B cell activation, the isolated cells were double-stained with FITC-conjugated antibodies against mouse CD3 (17A2), CD4 (G11.5), or CD19 (1D3) and phycoerythrin-conjugated anti-mouse CD69 antibodies (H1.2F3) (all from BD Biosciences) and analyzed by a flow cytometer (FACSCalibur, BD Biosciences) as described previously (32).

Spleenic T cell proliferation was measured by uptake of 3H-methyl thymidine (Amersham Pharmacia Biotech), the level of incorporated 3H-methyl thymidine being measured using a TopCount machine (Packard, PerkinElmer) as described previously (32).

NK cell activity was determined using a LIVE/DEAD Cell-Medi- cated Cytotoxicity kit (Molecular Probes) as described previously (7). In brief, 1 × 105 target cells (YAC-1 cells from a murine lymphoma) were prelabeled for 0.5 h with 1 μl of a 3 mM solution of DiOC18, and excess dye was removed by washing with PBS. The labeled YAC-1 cells were coincubated with 2 × 106 splenocytes for 4 h, and then 100 μl of 3.75 mM propidium iodine was added and the sample...
was incubated at 4°C for 20 min in the dark. NK cell activity was analyzed by flow cytometry and was calculated using the equation NK activity (%) = percentage of dead cells in the presence of NK cells – percentage of dead cells in the absence of NK cells.

Measurement of Serum Autoantibody and Complement Component Levels

The serum levels of anti-double stranded DNA (dsDNA) antibody (Alpha Diagnostic), C3 (Alpha Diagnostic), and C4 (USCN Life Science, Wuhan, China) were measured with an ELISA reader (Bio-Tek) at a wavelength of 450 nm using commercial ELISA kits according to the manufacturer’s instructions. For detection of serum antinuclear antibody (ANA), indirect immunofluorescence (IF) stain was performed in 1:40 diluted serum samples using HEP-2 slides (Binding Site, Birmingham, UK) as described previously (30).

Measurement of IL-6, IL-17A, and IL-18 mRNA

The total RNA of renal cortex RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions, and the expression levels of IL-6, IL-17A, or IL-18 were semiquantitively measured by real-time RT-PCR as described below. The primers sets were as follows: 5′-TGAAGTTCCTCTCTGCAAGAGACT-3′ and 5′-TGAAGTTCCTCTCTGCAAGAGACT-3′ for GAPDH; 5′-TCCACCGAATGGAAGACCTGATA-3′ and 5′-ACCAGCATCTTCTCGACCCCGCTGAAA-3′ for IL-17A; 5′-ACTGTACAACCGGAGTATC-3′ and 5′-ACTGTACAACCGGAGTATC-3′ for IL-6; 5′-TCCACCGAATGGAAGACCTGATA-3′ and 5′-ACCAGCATCTTCTCGACCCCGCTGAAA-3′ for IL-17A; 5′-ACTGTACAACCGGAGTATC-3′ and 5′-ACTGTACAACCGGAGTATC-3′ for IL-6; 5′-TCCACCGAATGGAAGACCTGATA-3′ and 5′-ACCAGCATCTTCTCGACCCCGCTGAAA-3′ for IL-17A; 5′-ACTGTACAACCGGAGTATC-3′ and 5′-ACTGTACAACCGGAGTATC-3′ for IL-6; 5′-TCCACCGAATGGAAGACCTGATA-3′ and 5′-ACCAGCATCTTCTCGACCCCGCTGAAA-3′ for IL-17A; 5′-ACTGTACAACCGGAGTATC-3′ and 5′-ACTGTACAACCGGAGTATC-3′ for IL-6; 5′-TCCACCGAATGGAAGACCTGATA-3′ and 5′-ACCAGCATCTTCTCGACCCCGCTGAAA-3′ for IL-17A; 5′-ACTGTACAACCGGAGTATC-3′ and 5′-ACTGTACAACCGGAGTATC-3′ for IL-6; 5′-TCCACCGAATGGAAGACCTGATA-3′ and 5′-ACCAGCATCTTCTCGACCCCGCTGAAA-3′ for IL-17A; 5′-ACTGTACAACCGGAGTATC-3′ and 5′-ACTGTACAACCGGAGTATC-3′ for IL-6; 5′-TCCACCGAATGGAAGACCTGATA-3′ and 5′-ACCAGCATCTTCTCGACCCCGCTGAAA-3′ for IL-17A; 5′-ACTGTACAACCGGAGTATC-3′ and 5′-ACTGTACAACCGGAGTATC-3′ for IL-6; 5′-TCCACCGAATGGAAGACCTGATA-3′ and 5′-ACCAGCATCTTCTCGACCCCGCTGAAA-3′ for IL-17A; 5′-ACTGTACAACCGGAGTATC-3′ and 5′-ACTGTACAACCGGAGTATC-3′ for IL-6; 5′-TCCACCGAATGGAAGACCTGATA-3′ and 5′-ACCAGCATCTTCTCGACCCCGCTGAAA-3′ for IL-17A; 5′-ACTGTACAACCGGAGTATC-3′ and 5′-ACTGTACAACCGGAGTATC-3′ for IL-6.

Data Analysis

The data were calculated and presented as means ± SE. Comparisons between two groups were performed using Student’s t-test. A P value <0.05 was considered statistically significant.

RESULTS

DCB-SLE1 Reduces Proteinuria, Hematuria, Renal Function Defects, and Severe Renal Lesions in the ASLN Model

Untreated ASLN mice showed increased urine protein levels starting at week 2 after ASLN induction, and these continued to rise until the mice were euthanized at week 5 (Fig. 1A); the mice also showed gross hematuria (dipstick test 3+) from week 4 to week 5 (Fig. 1B). These effects were significantly inhibited in the DCB-SLE1-treated ASLN (ASLN+DCB-SLE1) mice, although they still showed mild proteinuria compared with normal controls. The untreated ASLN mice also showed significantly increased serum levels of BUN (Fig. 1C) and Cr (Fig. 1D) at week 5, and again there was a dramatic improvement in renal function in the ASLN+DCB-SLE1 mice. At week 1, there was no significant difference in levels of BUN or Cr among the normal control, untreated ASLN mice, and ASLN+DCB-SLE1 mice.

As shown in Fig. 1E, at week 5 renal histopathology of the untreated ASLN mice revealed the characteristic severe lesions of lupus nephritis (4, 13, 57), we measured autoantibody (anti-dsDNA and ANA) and complement component (C3 and C4) in the serum and immune deposits in the kidney. As shown in Fig. 2A, serum anti-dsDNA antibody levels in untreated ASLN mice showed a significant increase as early as week 1 and a further increase at week 5 compared with normal controls but were significantly reduced in ASLN+DCB-SLE1 mice at both weeks 1 and 5. In parallel, as shown in Fig. 2B, although the serum from untreated ASLN mice gave a strong staining pattern at week 5, and a weaker pattern of staining at week 1, compared with the negative staining in normal control sera, the serum from ASLN+DCB-SLE1 mice generated a weak pattern of staining at week 5, and negative staining at week 1. As shown in Fig. 2C, untreated ASLN and ASLN+DCB-SLE1 mice showed no significant difference in serum C3 levels compared with normal controls at week 1. However, there was significant reduction in serum C3 levels in untreated ASLN mice at week 5 compared with normal controls, but this effect was suppressed by administration of DCB-SLE1.
Fig. 1. Proteinuria, hematuria, renal function, and severe renal lesions in the accelerated severe lupus nephritis (ASLN) model. A: time course studies of urine protein levels. B: time course studies of hematuria. C: serum blood urea nitrogen (BUN) levels at weeks 1 and 5. D: serum creatinine levels at weeks 1 and 5. E: kidney histopathological evaluation by hematoxylin and eosin staining. The arrowheads and black arrows indicate crescent formation and neutrophils, respectively. Original magnification, ×400. The semiquantitative analysis of kidney histopathological changes is shown at the bottom. Values are means ± SE for 6 mice/group. DCB-SLE1, extract of a mixture of 4 traditional Chinese medicinal herbs. See text for details. *P < 0.05, **P < 0.01, ***P < 0.005. #Not detectable.
in ASLN+DCB-SLE1 mice. There was no significant difference in serum C4 levels (Fig. 2D) among the normal controls, untreated ASLN, and ASLN+DCB-SLE1 mice.

Furthermore, at week 1 untreated ASLN mice showed a significant increase in IgG deposition in the glomerulus (Fig. 2E) and a slight but not significant increase in deposition of IgM (Fig. 2F) and C3 (Fig. 2G), with a much greater increase in all three at week 5. All of these effects were significantly reduced by DCB-SLE1 administration at both weeks 1 and 5.

**DCB-SLE1 Inhibits Renal Infiltration of T Cells, Monocytes/Macrophages, and Neutrophils**

Cellular immune effectors, such as T cells, macrophages, and neutrophils, play an important role in rapidly progressive glomerular nephritis (12, 56). T cells, which infiltrate the kidney and either cause direct cytotoxicity or recruit other inflammatory cells, such as monocytes/macrophages, play a crucial role in the pathogenesis of experimental and human lupus nephritis (36, 62). Since E. cortex, L. caulis, and H. diffusae Herba have been used as anti-inflammatory agents (21, 26, 66), we examined whether intrarenal infiltration of T cells, monocytes/macrophages, and neutrophils was suppressed by DCB-SLE1 administration. IHC studies at week 5 showed diffuse infiltration of T cells (CD3+, CD4+; and CD8+; Fig. 3, A–C), monocytes/macrophages (F4/80+; Fig. 3D), and neutrophils (CD11b+; Fig. 3E) into the periglomerular region of the renal interstitium in untreated ASLN mice, and these effects were significantly reduced by administration of DCB-SLE1 (all P < 0.005). At week 1, DCB-SLE1 administration caused a significant decrease in the infiltration of CD3+, F4/80+, and CD11b+ cells, and only a few, or no, CD4+ and CD8+ cells were seen in either untreated ASLN mice or ASLN+DCB-SLE1 mice.

**DCB-SLE1 Decreased IL-6, IL-17A, and IL-18 mRNA Levels in the Kidney**

IL-17, secreted by Th17 cells, is a cytokine with powerful inflammatory properties (11), and an enhanced Th17 cell response, seen as IL-6 overproduction, has been implicated in disease activity in patients with lupus nephritis (14, 40, 51). We therefore examined the effects of DCB-SLE1 on renal levels of mRNAs coding for IL-6 and IL-17A by real-time RT-PCR. At week 1, IL-6 mRNA levels in the kidney were significantly lower in ASLN+DCB-SLE1 mice than in untreated ASLN mice (Fig. 4A, P < 0.05), but there was no significant difference in IL-17A mRNA levels between the two groups (Fig. 4B). However, at week 5 a significant decrease in kidney levels of both mRNAs was seen in ASLN+DCB-SLE1 mice compared with untreated ASLN mice (Fig. 4, A and B, both P < 0.005).

IL-18 is involved in promoting the migration of circulating dendritic cells toward the kidney in active lupus nephritis (62). We have previously demonstrated that LPS-evoked IL-18 production leads to progression of the ASLN model (54). We therefore measured IL-18 mRNA levels in the kidney. As shown in Fig. 4C, real-time RT-PCR showed a significantly...
A–E: detection of CD3+ T cells (A), CD4+ T cells (B), CD8+ T cells (C), F4/80 monocytes/macrophages (D), and CD11b neutrophils (E) by immunohistochemical staining. Original magnification, ×400. The semiquantitative analysis is shown at the bottom right. Values are means ± SE for 6 mice/group. *P < 0.05, **P < 0.01, ***P < 0.005. #Not detectable.
increase in renal IL-18 mRNA levels as early as week 1 in untreated ASLN mice compared with normal controls \((P < 0.05)\) and an even greater increase at week 5 \((P < 0.005)\), and this large increase at week 5 was significantly inhibited in the ASLN+DCB-SLE1 mice \((P < 0.01)\), with a slight difference at week 1.

**DCB-SLE1 Inhibits Renal IL-6 and MCP-1 Protein Expression and NF-κB Activation**

IL-17 has been reported to recruit neutrophils and macrophages to inflammation sites by inducing overexpression of MCP-1 (11). We therefore analyzed protein expression of these cytokines in the kidney by IHC and found that expression of MCP-1 (Fig. 5A) and IL-6 (Fig. 5B) was significantly increased at week 1 in untreated ASLN mice compared with normal controls (MCP-1, \(P < 0.01\); IL-6, \(P < 0.05\)) and progressively increased at week 5 (both \(P < 0.005\)). In contrast, ASLN+DCB-SLE1 mice showed significantly reduced renal expression of these cytokines compared with untreated ASLN mice at both weeks 1 (both \(P < 0.05\)) and 5 (MCP-1, \(P < 0.005\); IL-6, \(P < 0.01\)).

We then measured NF-κB activation, since this results in expression of genes that encode these proteins (2, 67). As demonstrated by IHC in Fig. 6A, untreated ASLN mice showed significantly increased NF-κB p65 activity in the kidney compared with normal controls, as shown by its nuclear location in the glomeruli and tubulointerstitium and in mononuclear cells infiltrating the periglomerular regions, and DCB-SLE1 admin-

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**Fig. 4. Renal IL-6, IL-17A, and IL-18 mRNA levels. A–C: IL-6 (A), IL-17A (B), and IL-18 (C) mRNA levels measured by real-time RT-PCR. Values are means ± SE for 6 mice/group. \(*P < 0.05\), \(**P < 0.01\), \(***P < 0.005\).**

**Fig. 5. Intrarenal monocyte chemoattractant protein 1 (MCP-1) and IL-6 production. Detection of MCP-1 (A) or IL-6 (B) by immunohistochemical staining is shown. Original magnification, ×400. The semiquantitative score is shown on the right. Values are means ± SE for 6 mice/group. \(*P < 0.05\), \(***P < 0.005\).**
istration caused a significant decrease in NF-κB activation at both weeks 1 and 5 (both P < 0.005). Moreover, an ELISA-based NF-κB p65 activation assay demonstrated the similar pattern, although there was no significant difference among normal control, untreated ASLN, and ASLN/DCB-SLE1 mice at week 1 (Fig. 6B).

**DCB-SLE1 Decreases Serum Levels of Inflammatory Cytokines**

We then investigated whether DCB-SLE1 administration reduced systemic inflammation, as represented by serum levels of the inflammatory cytokines IL-6, MCP-1, IL-12p70, IFN-γ, TNF-α, IL-10, IL-17, and IL-18. Serum levels of IL-6 (Fig. 7A), MCP-1 (Fig. 7B), and IL-18 (Fig. 7G) were significantly increased in untreated ASLN mice compared with normal controls as early as week 1, and levels of all cytokines except IL-10 (Fig. 7F) progressively increased at week 5. In contrast, all of these effects were suppressed by administration of DCB-SLE1 in ASLN/DCB-SLE1 mice, with no significant difference in IL-10 levels. The serum levels of IL-17 were undetectable in all the mice.

**DCB-SLE1 Modulates Cellular Immune Responses**

T and B cell abnormalities and the generation of pathogenic autoantibodies have been demonstrated to play an important role in the progression of lupus nephritis (17). We therefore examined the effects of DCB-SLE1 on systemic cellular immunity using spleen tissues.

**Suppression of T/B cell activation.** As shown in Fig. 8, A and B, the percentage of CD3+CD69+ cells (activated T cells) in the spleen was significantly increased at both weeks 1 and 5 in untreated ASLN mice compared with normal controls (both P < 0.005), and this effect was prevented by the DCB-SLE1 administration at both weeks 1 and 5 (week 1, P < 0.05; week 5, P < 0.005). Similarly, the percentage of CD19+CD69+ cells (activated B cells) in ASLN mice was significantly reduced by DCB-SLE1 administration to the same levels as those in normal controls at weeks 1 and 5 (Fig. 8, C and D; week 1, P < 0.05; week 5, P < 0.005).

**Inhibition of T cell proliferation.** As shown in Fig. 8E, T cell proliferation in ASLN mice showed a dramatic increase at week 1, then tended to decrease at week 5, levels at both time points being significantly increased compared with those in normal control mice (both P < 0.005), and DCB-SLE1 administration significantly reduced T cell proliferation to the levels seen in normal control mice at both weeks 1 and 5 (week 1, P < 0.005; week 5, P < 0.01).

**Suppression of NK cell activity.** NK cells play an important role in the development of lupus in NZB/W F1 mice, since treatment with anti-NK1.1 monoclonal antibody ameliorates...
We therefore measured NK cell activity in the spleen. As shown in Fig. 8E, compared with normal control mice, NK cell activity in untreated ASLN mice was significantly increased at week 5, and this effect was significantly inhibited by administration of DCB-SLE1. At week 1, there was no significant difference in NK cell activity among normal control, untreated ASLN, and ASLN/H11001 DCB-SLE1 mice (Fig. 8F).

**DCB-SLE1 Prevents Apoptosis in the Spleen and Kidney**

Abnormal regulation of apoptosis and accumulation of apoptotic cells have been reported to enhance the progression of lupus nephritis (38, 50, 64), and marked apoptosis has been noted in the ASLN model (54). We therefore tested whether DCB-SLE1 administration prevented apoptosis in the spleen or kidney in ASLN mice, using the TUNEL assay. Although apoptosis was seen in the spleen of untreated ASLN mice, it was significantly suppressed by DCB-SLE1 administration (Fig. 9A). In parallel, a significant reduction in apoptosis was observed in the glomeruli and some renal tubules of ASLN/H11001 SLE1 mice compared with untreated ASLN mice, although a few apoptotic figures were still seen in the renal tubules of ASLN/H11001 SLE1 mice (Fig. 9B).

**DISCUSSION**

This study provides the first demonstration that DCB-SLE1, an extract of a mixture of *A. macrocephalae* Rhizoma, *E. cortex*, *L. caulis*, and *H. diffusae* Herba, can significantly ameliorate proteinuria, hematuria, renal function, and severity of pathology in the ASLN model. We propose that the following mechanisms are potentially responsible for its effectiveness in the mice ASLN model: 1) modulation of cellular and humoral immunity, including suppression of T/B cell activation and T cell proliferation and inhibition of NK cell activity; 2) inhibition of renal inflammation by suppression of Th1 and Th17 cytokine production and NF-κB activation; and 3) prevention of apoptosis in the kidney and lymphoid tissues (e.g., spleen).

It is well known that inappropriate or unbalanced T cell responses can initiate and perpetuate glomerular and tubulointerstitial tissue damage in severe lupus nephritis either directly by cytotoxic functions or cytokine secretion or indirectly by providing help for the production of autoantibodies and cytokines and the generation of immune complexes or by activating macrophages and neutrophils (58, 63). We propose that the key mechanism responsible for the effects of DCB-SLE1 in the ASLN model is the systemic modulation of T/B cell activation and/or proliferation. We showed that DCB-SLE1 administration significantly inhibited T/B cell activation and T cell proliferation in ASLN+DCB-SLE1 mice as early as week 1 and that these effects were maintained up to at least week 5. These effects were closely associated with a significant reduction in serum autoantibody levels and glomerular immune deposits.

NK cells develop in lymphoid tissue and can be found in close proximity to T cells, where they can affect adaptive immune responses (39), with possible implications for autoimmune-adaptive responses (45). Although the role of NK cells in disease promotion or protection from autoimmune disease is not clear, previous studies have described impaired NK cytotoxicity in systemic lupus erythematosus patients (20, 25). Moreover, in a mouse model of autoimmune myasthenia gravis, Shi et al. (53) showed that depletion of NK cells during the priming phase protects the mice from disease development by reducing IFN-γ production by CD4+ T cells and increasing the production of the anti-inflammatory cytokine transforming growth factor-β, while levels of pathogenic antibodies decreased. In the present study, we demonstrated that DCB-SLE1 administration significantly inhibited the increase in NK cell activity in ASLN mice at week 5 and significantly suppressed serum levels of IFN-γ. These results suggest that the effects of DCB-SLE1 on NK cell activity may contribute to the attenuation of ASLN.

![Fig. 7. Serum levels of inflammatory cytokines.](http://ajprenal.physiology.org/)

**Fig. 7.** Serum levels of inflammatory cytokines. A: IL-6. B: MCP-1. C: IL-12p70. D: IFN-γ. E: TNF-α. F: IL-10. G: IL-18. Values are means ± SE for 6 mice/group. *P < 0.05, **P < 0.01, ***P < 0.005.
The effects of Th17 cells, characterized by the production of IL-17, that are potentially relevant to rapidly progressive glomerular nephritis include direct effects on neutrophils and macrophages (56). IL-17 has a potent proinflammatory effect and recruits neutrophils and macrophages to the site of inflammation via overexpression of IL-8 and MCP-1 (44, 52). Similarly, IL-18 has been shown to prolong neutrophil survival in patients (1) and in vitro (9). Furthermore, IL-18 is considered to mediate inflammation via the initiation and expansion of Th1 responses, as well as having direct effects on macrophages and lymphocytes (8, 23), and is nephritogenic, since it recruits IL-18R$^+$ dendritic cells to the kidney, thereby contributing to immune system-mediated renal damage (8, 59, 61). We have previously demonstrated that IL-18 produced by mesangial cells contributes to the severe glomerular lesions in the ASLN model, including neutrophil infiltration, intrinsic cell prolifera-

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**Fig. 8. Systemic immunity.** A–D: immunofluorescence dot-plot pattern of the CD69 activation marker on CD3$^+$ T cells (A) or on CD19$^+$ B cells (C) on splenocytes and the percentage of CD3$^+$CD69$^+$ T cells (B) and CD19$^+$CD69$^+$ B cells (D). E: T cell proliferation. F: NK cell activity. Values are means ± SE for 6 mice/group. *P < 0.05, **P < 0.01, ***P < 0.005.
In the present study, we showed that DCB-SLE1 administration significantly reduced serum levels of IFN-γ/H9253, IL-6, IL-12, and IL-18 and renal levels of IL-6, IL-17A, and IL-18 mRNAs in the ASLN model, suggesting that DCB-SLE1 might benefit the animals, in part, by blocking Th1- and Th17-biased responses. Our data showed that DCB-SLE1 inhibit neutrophil infiltration into the kidney in part by a dramatic reduction in renal IL-17A and IL-18 mRNAs expression. We also demonstrated that DCB-SLE1 administration significantly inhibited infiltration of T cells, mono-

Fig. 9. Apoptosis in the kidney and spleen. A and B: apoptosis was detected in the spleen (A) and kidney (B) by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL). Original magnification, ×400. C and D: scoring of apoptosis-positive cells in the spleen (C) and kidney (D). Values are means ± SE for 6 mice/group. *P < 0.05, **P < 0.01, ***P < 0.005.
cytes/macrophages, and neutrophils into the kidney and blocked NF-κB activation in the kidney, contributing to the prevention of interstitial inflammation. These effects, operating locally in the kidney to suppress inflammatory cell infiltration, might serve as an immediate and crucial mechanism explaining the beneficial therapeutic effects of DCB-SLE1 in ASLN.

Besides, we showed that, as early as week 1, DCB-SLE1 administration inhibited the increase in serum levels of IL-6 and IL-18 and kidney levels of IL-6 and MCP-1 in untreated ASLN mice. In accordance with this effect, we (29) recently demonstrated the mesangial cells from NZB/W F1 mice exhibited a significantly augmented activation of the Toll-like receptor 4-MyD88-NF-κB signaling pathway, and its resultant significantly enhanced production of MCP-1, OPN, and IL-6 compared with the mesangial cells from their wild-type mice. This finding further confirms the potential effect of DCB-SLE1 that might act by blocking the early proinflammatory responses to LPS. Huugen et al. (27) reported that intraperitoneal administration of LPS can induce an immediate, transient rise in serum levels of TNF-α as early as 1 h after injection, and then the TNF-α levels are undetectable at days 1 and 6 after administration. In the present study, although we failed to show the significant difference in the serum TNF-α levels among the normal controls, untreated ASLN mice, and ASLN+DCB-SLE1 mice at week 1, it might not necessarily exclude the early inhibitory effects of DCB-SLE1 on TNF-α production. Instead, in the present study, 1 wk after LPS injection might be too late to determine the transient rise of serum TNF-α levels, and further mechanistic studies on the time course of the effects of DCB-SLE1 on TNF-α production are warranted.

Although plant-derived agents could be used alone, combined extracts have been reported to allow a reduction in side effects, possibly leading to an increase in effectiveness (37). Besides, in a preliminary study, we had tested different compositions in lupus-prone NZB/W F1 mice and found four herbal medications showed a potential activity for lupus treatment (data not shown). In addition, we found that the extract of a mixture of these four herbal medications contains oleaolic acid and ursolic acid, which are triterpene acids and have numerous pharmacological activities including anti-inflammatory, anticancer, and hepatoprotective effects (33). In the present study, we showed that DCB-SLE1 might appear to have broad immunomodulatory potential. In this regard, oleaolic acid exhibits inhibitory effects on carageenan-induced rat paw edema and formalin-induced arthritis (18) and inhibits the LTB4 production in rat peritoneal leukocytes (19). In addition, ursolic acid shows a significant inhibitory effect on cyclooxygenase-2 transcription in PMA-treated human mammary and oral epithelial cells (55). However, further mechanistic studies on whether specific activities can be assigned to specific components of the mixture are warranted.

Finally, we (32) and others (49) have proposed that the use of apoptosis inhibitors might prevent severe renal injury in lupus nephritis. We have previously demonstrated that LPS-evoked IL-18 production is associated with inflammatory cell infiltration into the glomeruli and leads to apoptosis in the ASLN model (54). In the present study, we showed that DCB-SLE1 protected against both systemic (spleen) and local (kidney) apoptosis. Our data showed that the blocking of apoptosis in the kidney was associated with a lower histological severity of the renal lesions in ASLN. As a result, inhibition of apoptosis in lymphoid tissues (as represented by the spleen) and in the kidney may contribute to the beneficial effects of DCB-SLE1 in ASLN.

In the present study, we administered the mixture to the animals before disease induction, and this might be a potential problem in its applicability to human disease. Based on 1) the fact that DCB-SLE1 altered T/B cell activity and prevented apoptosis in ASLN mice and 2) beneficial effects were observed in a spontaneous lupus nephritis model in NZB/W F1 mice by treatment with DCB-SLE1 (data not shown), we speculate DCB-SLE1 might be a drug that could be used to maintain lupus in remission. However, further studies to evaluate the effects of giving DCB-SLE1 after disease establishment are warranted.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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