

## Curcumin attenuates lupus nephritis upon interaction with regulatory T cells in New Zealand Black/White mice

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### Abstract

Curcumin has been used in Asian traditional medicine for its medicinal properties. Recent studies have demonstrated that curcumin has antioxidant, anti-tumour and anti-inflammatory activities. The aim of the present study is to investigate the effects of curcumin on established lupus nephritis (LN) in New Zealand Black/White (NZB/W) F1 female mice, in particular, its interaction with regulatory T ( $T_{\text{reg}}$ ) cells. Starting at 18 weeks of age, mice were fed a standard diet or a diet containing 1% curcumin until the end of the study. The proteinuria level and the serum levels of IgG1, IgG2a and anti-double-stranded DNA (dsDNA) IgG antibodies were measured. Additionally, IgG immune complex deposition in the glomeruli and renal inflammation were compared between curcumin-treated mice and control mice. Curcumin decreased the proteinuria level and serum levels of IgG1, IgG2a and anti-dsDNA IgG antibodies in NZB/W F1 female mice. IgG immune complex deposition in the glomeruli was reduced in curcumin-treated mice. Furthermore, renal inflammation was also decreased after curcumin treatment. Interestingly, these therapeutic effects of curcumin disappeared after  $T_{\text{reg}}$  depletion by anti-CD25 antibody injection. Curcumin exerted a protective effect against LN in NZB/W F1 mice. We speculate that the protective effects of curcumin in LN may involve, at least in part, its interaction with  $T_{\text{reg}}$  cells.

**Key words:** Autoimmunity; Lupus nephritis; New Zealand Black/White F1 mice; Curcumin; Regulatory T cells

Systemic lupus erythematosus is an autoimmune disorder affecting multiple organ systems, including the kidney, skin, lung, heart, haematopoietic system and brain<sup>(1)</sup>. Lupus nephritis (LN) is a frequent and serious complication of systemic lupus erythematosus that is directly related to the morbidity and mortality of patients<sup>(2)</sup>.

The production of autoantibodies against the nucleus and formation of glomerular immune deposits play a crucial role in the pathogenesis of LN and the local production of both cytokines and chemokines triggers glomerular inflammation, which ultimately leads to irreversible renal damage<sup>(3,4)</sup>. The interaction between B and T cells plays an especially dominant role in the progression of LN, leading to renal failure<sup>(5)</sup>. B cells are often seen as the central cause of disease pathogenesis, which they induce by secreting autoantibodies<sup>(6)</sup>. T cells are considered critical for the provision of help to B cells in the production of autoantibodies<sup>(7)</sup>. Therefore, the therapeutic target of LN has been suppression of the over-activated immune system via the use of high-dose glucocorticoids and immunosuppressants. However, only about 80% of patients achieve renal remission in response to initial therapy using

current treatment protocols; while approximately 30% experience renal flares, 5–20% of patients progress to end-stage kidney disease and treatment-related toxicity is appreciable<sup>(8)</sup>. Thus, there is a need for alternative therapeutic options.

Recently,  $CD4^+CD25^+$  regulatory T ( $T_{\text{reg}}$ ) cells have been shown to play a critical role in the maintenance of tolerance in the immune system<sup>(9–11)</sup>. In addition, there is a great deal of convincing evidence that  $T_{\text{reg}}$  cells are impaired in their suppressive function in patients with autoimmune diseases such as rheumatoid arthritis<sup>(12)</sup>, multiple sclerosis<sup>(13)</sup> and lupus<sup>(14)</sup>.

Curcumin is a polyphenolic compound derived from the rhizomes of the plant *Curcuma longa*, which is used in Asian traditional medicine for its medicinal properties<sup>(15)</sup>. The medicinal value of curcumin has been well recognised owing to its antioxidant, anti-tumour and anti-inflammatory activities. Additionally, recent studies have demonstrated that curcumin has the potential for use in the treatment of animal autoimmune disease models such as experimental allergic encephalomyelitis<sup>(16)</sup> and inflammatory bowel disease<sup>(17)</sup>. These findings strongly suggest that curcumin may have

**Abbreviations:** dsDNA, double-stranded DNA; Foxp3, forkhead box P3; GN, glomerulonephritis; LN, lupus nephritis; MCP-1, monocyte chemoattractant protein-1; NZB/W, New Zealand Black/White;  $T_{\text{reg}}$ , regulatory T.

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a therapeutic effect on LN. In addition, recent clinical research demonstrated that oral supplementation of turmeric showed beneficial effects in LN patients<sup>(18)</sup>. However, the specific effect of curcumin on LN has not been well examined to date.

The present study was conducted to investigate the effects of curcumin on autoimmunity using New Zealand Black/White (NZB/W) F1 female mice. NZB/W mice spontaneously develop autoimmune disease as they age, which is characterised by lethal immune complex glomerulonephritis (GN), proteinuria and renal dysfunction. These symptoms in NZB/W mice closely resemble lupus in human subjects with severe GN<sup>(19)</sup>. Therefore, we evaluated curcumin to determine if it could ameliorate renal inflammation, including immune complex-mediated GN with proteinuria and antinuclear autoantibody production. We found that renal disease improved remarkably in curcumin-treated mice when compared with control mice.

## Materials and methods

### Mice

NZB/W F1 female mice were purchased from Japan SLC, Inc. NZB/W F1 female mice spontaneously develop an autoimmune disease with immune complex GN, proteinuria and progression to renal insufficiency<sup>(19)</sup>. All mice were housed under specific pathogen-free conditions during the experiments, which were conducted according to the ethical principles and guidelines established by Kyung Hee University for the care and use of experimental animals. At 18 weeks of age, mice were given curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione), which was mixed in a normal diet at a concentration of 1% (w/w). This dose of curcumin was selected based on previous studies in a murine skin carcinogenesis model<sup>(20)</sup>. All feeds were pelleted to avoid stratification and to ensure uniform feed and curcumin intake in the treated animals. All control and experimental diets were prepared every 2 weeks in our laboratory and stored at  $-20^{\circ}\text{C}$  until use.

### Measurement of proteinuria

Beginning at 18 weeks of age, proteinuria was measured semi-quantitatively by impregnating wool paper test strips with spot urine (URISCAN; Youngdong Pharmaceutical Company). The colour change of the strip infiltrated with the urine sample was visually judged against a standard strip and scored from 0 to 5+, according to the manufacturer's instructions. The urinary protein content was graded according to the score as follows: 0 =  $<100\text{ mg/l}$ , 1+ =  $100\text{ mg/l}$ , 2+ =  $300\text{ mg/l}$ , 3+ =  $1000\text{ mg/l}$ , 4+ =  $3000\text{ mg/l}$  and 5+ =  $10\,000\text{ mg/l}$ .

### Sera collection and autoantibody determination

At 18 and 32 weeks of age, mice were bled from the retro-orbital sinus following inhalation of isoflurane anaesthesia. A serum Ig class-specific antibody assay was conducted

using mouse IgG1, IgG2a, IgG2b and IgG3 (Bethyl Laboratories, Inc.) ELISA kits. The serum anti-double-stranded DNA (dsDNA) IgG was evaluated using ELISA kits purchased from Alpha Diagnostic International at 32 weeks of age. All ELISA were conducted according to the manufacturer's instructions.

### Immunofluorescent staining of IgG

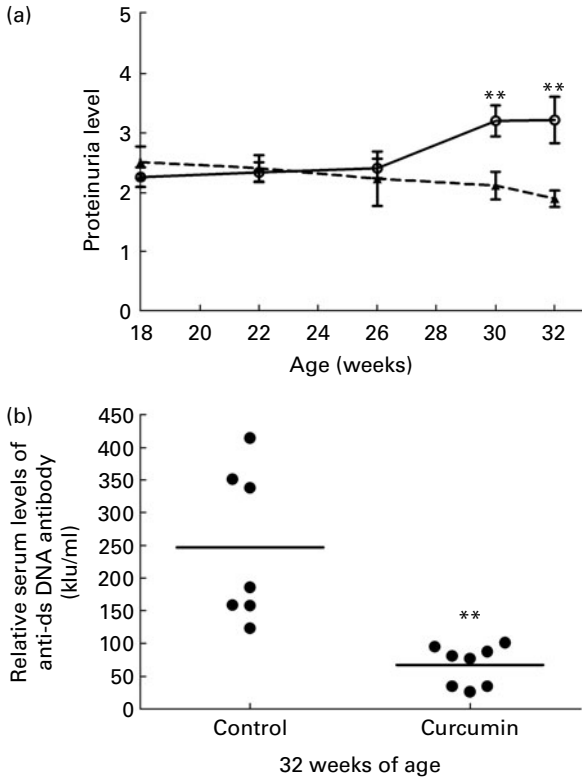
Kidneys were embedded in Tissue-Tek OCT compound (Sakura), rapidly frozen using a Freezer Spray (Thermo Electron) and then cut into  $20\text{ }\mu\text{m}$ -thick sections with a cryostat. The sections were subsequently permeabilised for 20 min in PBS containing 0.5% Triton X-100, after which the non-specific reactions were blocked and the samples were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (SantaCruz Biotechnology, 1:100) antibody at the optimal concentration. Finally, the fluorescence intensities within the glomeruli were measured using a confocal laser scanning microscope system (LSM 5 PASCAL, Carl Zeiss). Kidney sections were examined in each mouse, and at least ten kidney sections were analysed using the manufacturer's software (LSM 5 PASCAL release version 4.0 SP2; Carl Zeiss).

### Histology and morphometric evaluation

At 32 weeks of age, the mice were killed for pathological evaluation. One-half of the left kidney from each mouse was fixed in 4% paraformaldehyde solution, dehydrated in alcohol and embedded in paraffin. The kidney blocks were then cut into  $5\text{ }\mu\text{m}$ -thick sections and stained with periodic acid–Schiff reagents. A total of twenty-five sequential glomeruli from the superior, middle and inferior cortices of each kidney were scored for damage by a pathologist in a single-blind fashion on a scale of 0–4+, where 0 = normal, 1+ = focal, mild or early proliferative, 2+ = multifocal proliferative with increased matrix and inflammatory cells, 3+ = diffuse proliferative and 4+ = extensive sclerosis/crescents.

### Kidney TNF- $\alpha$ , IL-6 and monocyte chemoattractant protein-1

To examine pro-inflammatory molecules that were generated by nephritis, TNF- $\alpha$ , IL-6 and monocyte chemoattractant protein-1 (MCP-1) were measured in the mouse kidneys using ELISA (BD Biosciences). Briefly, the snap-frozen half of the left kidney tissue was homogenised in a PRO-PREP protein extraction solution (iNTRON Biotechnology, Inc.), incubated on ice for 20 min and then centrifuged at  $13\,000\text{ rpm}$  ( $4^{\circ}\text{C}$ ) for 15 min. The supernatant was used for a kidney pro-inflammatory cytokine assay. The protein concentrations in each supernatant were determined by a BCA<sup>TH</sup> Protein Assay Kit (Thermo Scientific). The protein levels of the cytokines were corrected for the total amount of protein, after which the results were expressed as  $\text{pg/mg}$  or  $\text{ng/mg}$ .



**Fig. 1.** Effect of curcumin (⊖) on proteinuria and anti-double-stranded DNA (dsDNA). (a) Protein urea in New Zealand Black/White F1 mice at different weeks of age. (b) Anti-dsDNA IgG antibody was measured at 32 weeks of age. Values are means, with their standard errors represented by vertical bars ( $n$  7–8). \*\*Mean value was significantly different from that of control mice (▲) ( $P < 0.01$ ; Student's  $t$  test).

### Isolation of RNA and real-time PCR

Total cellular RNA was extracted using TRIzol reagent (Invitrogen Life, Technologies), according to the manufacturer's protocols, after which the concentration of total RNA was quantified by determining the optical density at 260 nm. Complementary DNA was synthesised using Transcriptor First Strand cDNA Synthesis Kit (Roche), according to the manufacturer's protocols, and then stored at  $-20^{\circ}\text{C}$ . Real-time quantitative PCR was conducted using LightCycler<sup>®</sup> 480 (Roche), employing SYBR Green I (Roche) as the

dsDNA-specific binding dye for continuous fluorescence monitoring. Amplification was carried out using the reaction mixture, with a total volume of 20  $\mu\text{l}$ , containing 375 nm of each gene-specific primer, 2  $\times$  PCR Master Mix (Applied Biosystems) and 2  $\mu\text{l}$  of complementary DNA. The mixtures were then subjected to forty cycles of denaturation ( $95^{\circ}\text{C}$ , 30 s), annealing and extension at  $60^{\circ}\text{C}$ , with fluorescence being measured at the end of each cycle. After the cycles were terminated, the signals at temperatures between 60 and  $95^{\circ}\text{C}$  were also collected to generate a dissociation curve. All reactions were conducted in duplicate to confirm reproducibility and a negative control was included (without template) to verify that no primer dimers were being generated. A standard curve for each primer was constructed using serial dilutions of complementary DNA, and the amount of target mRNA in each sample was normalised using that of the mean glyceraldehyde-3-phosphate dehydrogenase levels. The primer sequences are listed in Table 2.

### Depletion of $\text{CD4}^+\text{CD25}^+$ regulatory T cells in vivo

Anti-mouse CD25 rat IgG1 (anti-CD25; clone PC61) were generated in-house from hybridomas obtained from American Type Culture Collection. A dose of 0.5 mg of anti-CD25 antibody was injected twice a week until the end of the experiment. The efficacy of  $\text{CD4}^+\text{CD25}^+$   $\text{T}_{\text{reg}}$  cell depletion was confirmed by flow cytometry analysis, using phycoerythrin-anti-mouse CD25 and FITC-anti-mouse CD4 (eBioscience).

### Statistical analysis

Statistical analysis of the data was conducted using Prism 4.02 software (GraphPad Software, Inc.). Statistical analysis was conducted using Student's  $t$  test. Results with a  $P$  value  $< 0.05$  were considered statistically significant.

## Results

### Outcome

Initially, ten mice were included in each group; however, three mice in the control group, two mice in the curcumin group, four mice in the anti-CD25 control group and three mice in the anti-CD25 curcumin group were dead before

**Table 1.** Effect of curcumin on serum IgG isotypes† (Mean values with their standard errors,  $n$  7–8)

Isotypes	18 weeks				32 weeks			
	Control		Curcumin		Control		Curcumin	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
IgG1 ( $\mu\text{g/ml}$ )	913.1	100.5	655.2	70.17	1792	237.3	1093*	79.45
IgG2a ( $\mu\text{g/ml}$ )	1452	58.32	1464	70.94	2716	62.78	671.3***	44.52
IgG2b ( $\mu\text{g/ml}$ )	58.89	3.503	44.81	3.349	142.6	32.55	188.2	25.98
IgG3 ( $\mu\text{g/ml}$ )	182.9	25.34	197.1	57.80	1048	179.2	925.5	113.7

Mean value was significantly different from that of control mice at 32 weeks of age: \*  $P < 0.05$ , \*\*\*  $P < 0.001$  (Student's  $t$  test).  
† Serum IgG1, IgG2a, IgG2b and IgG3 were measured at 18 and 32 weeks of age.

**Table 2.** Primer sequences

Primer	Forward	Reverse
Foxp3	5'-AGC CTG CTC CAT ACC TTG AA-3'	5'-GCC CAA GAT GTG CAC TGATA-3'
T-bet	5'-GCC AGG GAA CCG CTT ATA TG-3'	5'-GAC GAT CAT CTG GGT CAC AT-3'
GATA-3	5'-GAA GGC ATC CAG ACC CGA AA-3'	5'-ACC CAT GGC GGT GAC CAT GC-3'
GAPDH	5'-TTCACC ACC ATG GAG AAG GC-3'	5'-GGC ATG GAC TGTGGT CAT GA-3'

Foxp3, forkhead box P3; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

reaching 32 weeks of age. Data from these mice were excluded from the analysis of treatment efficacy. NZB/W mice treated with curcumin gained weight in a manner similar to that of mice provided with the normal diet (at 32 weeks of age, body weight: control 32.00 (SEM 0.98) g *v.* curcumin 34.78 (SEM 0.78) g). There were no signs of liver dysfunction in treated mice, as indicated by levels of serum aspartate aminotransferase and alanine aminotransferase being similar to the normal diet (at 32 weeks of age, aspartate aminotransferase: control 208.3 (SEM 54.27) IU/l *v.* curcumin 127.1 (SEM 17.93) IU/l; alanine aminotransferase: control 57.00 (SEM 19.81) IU/l *v.* curcumin 36.43 (SEM 4.54) IU/l).

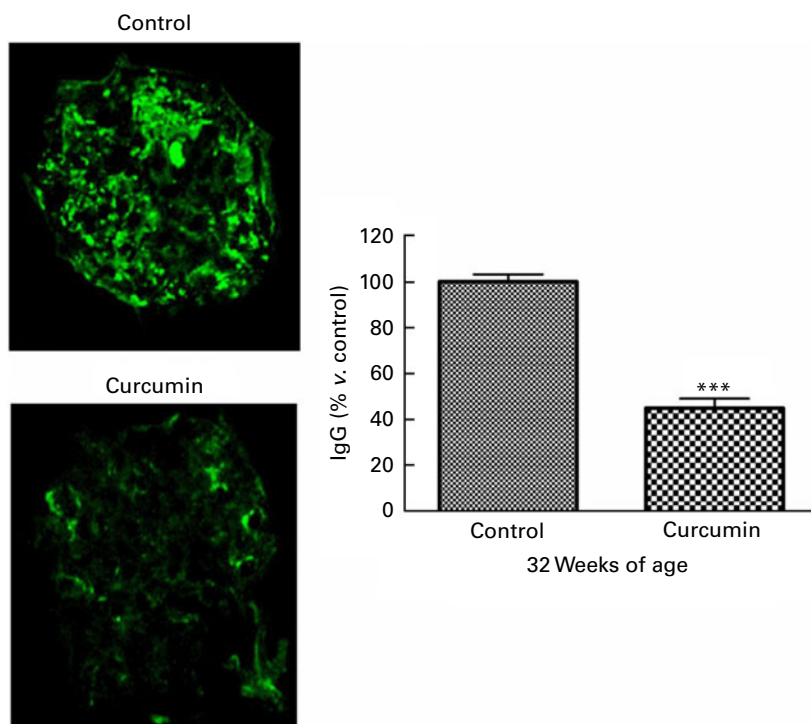
#### Effect of curcumin on proteinuria

Significant proteinuria ( $\geq 2+ = 300$  mg/l) was detected in 100% of the mice in all groups prior to the start of the treatment. At 26 weeks of age, the mean level of urinary protein did not differ significantly between the two groups.

At 30 weeks of age, the control group developed severe proteinuria. Conversely, the mean level of urinary protein in the curcumin-treated group began to decrease (control 3.2 (SEM 0.26) *v.* curcumin 2.1 (SEM 0.23);  $P < 0.01$ ). By the end of the study, renal function had progressively deteriorated in the control group. In addition, urinary protein excretion was significantly attenuated in curcumin-treated mice (Fig. 1(a); control 3.2 (SEM 0.39) *v.* curcumin 1.9 (SEM 0.14);  $P < 0.01$ ).

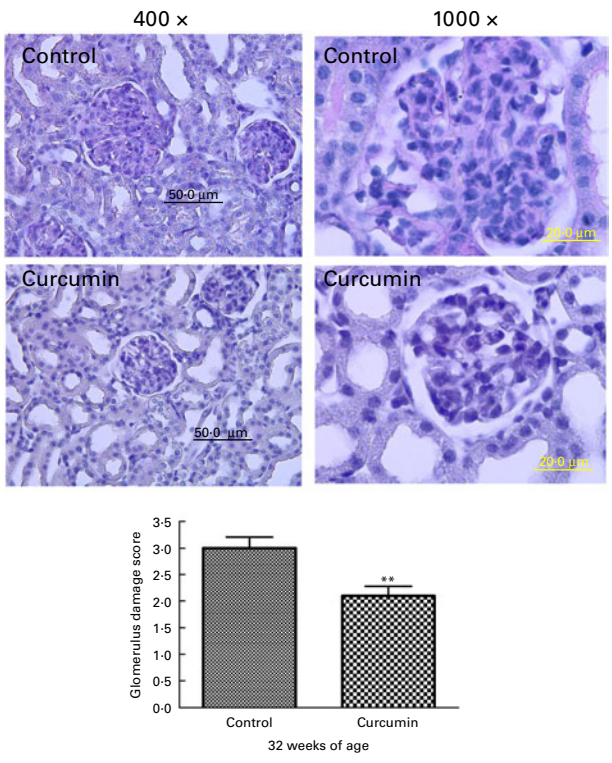
#### Effect of curcumin on serum anti-double-stranded DNA IgG

The serum level of anti-dsDNA IgG was also determined at 32 weeks of age. Mice fed the normal diet had very high levels of anti-dsDNA antibodies in their serum. Anti-dsDNA antibody levels were diminished in the curcumin-treated mice (Fig. 1(b); control 250 (SEM 44) kIU/ml *v.* curcumin 67 (SEM 11) kIU/ml;  $P < 0.01$ ).



**Fig. 2.** (colour online) Effect of curcumin on immune complex deposition in the glomeruli. Renal glomeruli were evaluated by staining kidney cryosections with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG antibody. Fluorescence intensities within the glomerular capillary walls were detected using confocal microscopy. At least ten glomeruli per section were analysed (magnification:  $\times 400$ ). Values are means, with their standard errors represented by vertical bars ( $n 7-8$ ). \*\*\* Mean value was significantly different from that of control mice ( $P < 0.001$ ; Student's *t* test).





**Fig. 3.** (colour online) Effect of curcumin on glomerulonephritis. Renal glomeruli from saline-treated mice and curcumin-treated mice (magnification:  $\times 400$  or  $\times 1000$ ). A total of twenty-five sequential glomeruli from the superior, middle and inferior cortices of each kidney were scored for damage by a pathologist in a single-blind fashion. Values are means, with their standard errors represented by vertical bars ( $n 7-8$ ). \*\*Mean value was significantly different from that of control mice ( $P < 0.01$ ; Student's *t* test).

*Effect of curcumin on serum IgG isotypes*

Serum levels of circulating IgG1, IgG2a, IgG2b and IgG3 were measured at 18 and 32 weeks of age. As expected, the levels of IgG1, IgG2a, IgG2b and IgG3 were significantly up-regulated in the control group at 32 weeks of age. However, the levels of IgG1 and IgG2a were significantly lower at the end of the treatment period in curcumin-treated mice when compared with the control mice. However, the levels of IgG2b and IgG3 were not significantly different between the groups (Table 1).

*Effect of curcumin on immune complex deposits*

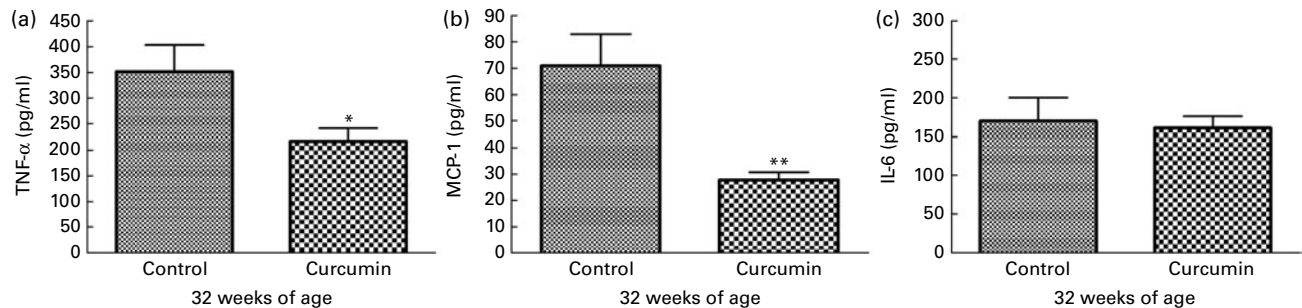
In LN, immune complex accumulation is believed to initiate inflammation. To understand how curcumin decreases GN, we assessed glomerular IgG binding by immunofluorescence microscopy. As shown in Fig. 3, a marked deposition of IgG was observed in the renal glomeruli of the control group. Blinded scoring of IgG staining intensity revealed increased IgG staining in the saline group when compared with the curcumin group, indicating that the amount of immune deposits present in the glomeruli was correlated with the damage observed by light microscopy (Fig. 2; control 100 (SEM 3.2) *v.* curcumin 45 (SEM 4.1);  $P < 0.001$ ).

*Effect of curcumin on renal histology*

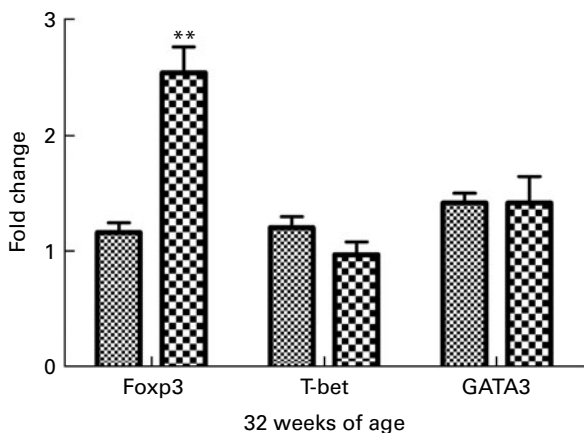
To evaluate the effects of curcumin on the development of GN, all surviving mice were killed at 32 weeks, after which kidney sections were examined by light microscopy. Glomeruli from the control group showed chronic GN, i.e. structural damage, including hyaline lesions, fibrinoid necrosis, cellular crescents, mesangial proliferation and mesangiolysis. In contrast, many glomeruli from the curcumin group showed mildly abnormal histology. To quantify these changes, we conducted blinded scoring of 'glomerular damage'. The curcumin diet significantly reduced glomerular damage when compared with the normal diet (Fig. 3).

*Effect of curcumin on renal TNF- $\alpha$ , IL-6 and monocyte chemoattractant protein-1*

To examine pro-inflammatory molecules that were generated by nephritis, TNF- $\alpha$ , IL-6 and MCP-1 were measured in the kidneys of mice from each group. Control group mice showed increased levels of TNF- $\alpha$  and MCP-1 at 32 weeks of age. In contrast, the curcumin group showed reduced TNF- $\alpha$  (control 352.1 (SEM 51.51) pg/mg and curcumin 203.4 (SEM 15.10) pg/mg;  $P < 0.05$ ) and MCP-1 (control 70.99 (SEM 11.98) pg/mg and curcumin 28.51 (SEM 3.176) pg/mg;  $P < 0.01$ ) levels (Fig. 4). However, there was no significant difference in IL-6 level at 32 weeks of age.



**Fig. 4.** Effect of curcumin on (a) TNF- $\alpha$ , (b) monocyte chemoattractant protein-1 (MCP-1) and (c) IL-6 in kidney. The curcumin group showed a significantly reduced increase in TNF- $\alpha$  and MCP-1 when compared with the control group. Values are means, with their standard errors represented by vertical bars ( $n 7-8$ ). Mean values were significantly different from that of control mice: \*  $P < 0.05$ , \*\*  $P < 0.01$  (Student's *t* test).



**Fig. 5.** Real-time PCR analysis of spleen in New Zealand Black/White mice. Real-time PCR was conducted in a Roche LightCycler® 480 (Roche) using SYBR Green I as the double-stranded DNA-specific binding dye and continuous fluorescence monitoring. Values are means, with their standard errors represented by vertical bars ( $n$  3). \*\*Mean value was significantly different from that of control (▨) mice ( $P < 0.01$ ; Student's  $t$  test). ▩, Curcumin.

*Effect of curcumin on T helper 1/T helper 2/T<sub>reg</sub>-specific transcription factor expression*

To determine if curcumin treatment influenced helper T cell lineage development, we conducted real-time RT-PCR analysis of spleens isolated from 32-week-old mice fed a 1% curcumin

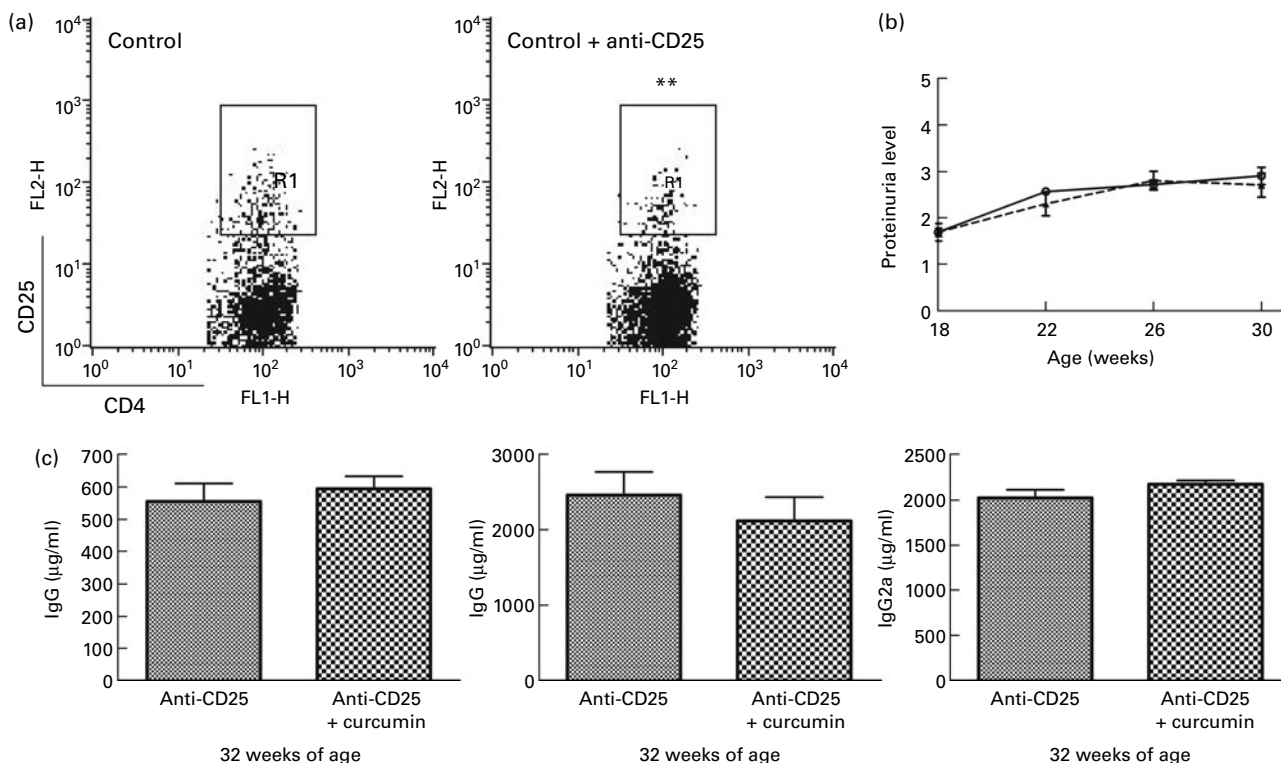
diet or normal diet for forkhead box P3 (Foxp3), T-bet and GATA-3. It is well-known that Foxp3 is the key transcription factor controlling Treg cell development and function<sup>(21)</sup>. T-bet is a transcription factor directing T helper 1 lineage commitment<sup>(22)</sup>. GATA-3 is a transcription factor maintaining T helper 2 cell function<sup>(23)</sup>. The results showed that Foxp3 expression was significantly increased in the curcumin group ( $P < 0.01$ ,  $n$  3) and the T-bet expression tended to be lower than in the control group (Fig. 5).

*Effect of curcumin on regulatory T-depleted lupus-prone mice*

Based on the Foxp3 mRNA expression, T<sub>reg</sub> cells appeared to have an extensive relationship with the therapeutic effect of curcumin in lupus-prone mice. We depleted T<sub>reg</sub> in NZB/W mice using PC61 and examined proteinuria and serum IgG isotype levels. Interestingly, the beneficial effects of curcumin disappeared (Fig. 6).

**Discussion**

In the present study, we explored the therapeutic effects of curcumin on established LN in NZB/W F1 mice. Curcumin ameliorated the progression of renal diseases in NZB/W F1 mice. The proteinuria level and the serum levels of IgG1, IgG2a and anti-dsDNA IgG antibodies in the curcumin



**Fig. 6.** Effect of anti-CD25 on New Zealand Black/White (NZB/W) mice. (a) A dose of 0.5 mg of anti-CD25 antibody was injected twice a week until the end of the experiment. The efficacy of CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cell depletion was confirmed by flow cytometry analysis. Gated on CD4<sup>+</sup>T cell (control  $n$  3 (8.1 (SEM 0.11)%), anti-CD25  $n$  15 (5.4 (SEM 0.39)%). \*\*Mean value was significantly different from that of control mice ( $P < 0.01$ ). (b) Protein urea in NZB/W F1 mice at different weeks of age. (c) Serum IgG, IgG1 and IgG2a were detected at 32 weeks of age (anti-CD25 (▨)  $n$  7, anti-CD25 + curcumin (▩)  $n$  8). Values are means, with their standard errors represented by vertical bars. Data were analysed by Student's  $t$  test. FL1-H, fluorescence level 1-height; FL2-H, fluorescence level 2-height; R1, region 1.

group were lower than those in the control group. Concomitant with serum IgG levels, IgG immune complex deposition in the glomeruli was reduced in curcumin-treated mice. Furthermore, renal inflammation was also decreased after curcumin treatment.

Hereditary lupus of NZB/W mice is an antibody-mediated systemic autoimmune disease in which T cells play a critical role in augmenting autoantibody secretion by B cells<sup>(7)</sup>. It is well known that when B cells come into contact with T helper 1 cells, they secrete IgG2a and IgG3<sup>(24)</sup>, but that upon interaction with T helper 2 cells, they produce IgG1 isotypes<sup>(25)</sup>. The present findings demonstrated that NZB/W mice treated with curcumin showed decreased levels of IgG1 and IgG2a. The production of IgG3 was slightly down-regulated. These results indicate that curcumin exerts immunosuppressive effects on both T helper 1 and T helper 2 immune responses and suggest that CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells could be involved in this suppressive effect of curcumin. Indeed, the present study demonstrated that NZB/W mice treated with curcumin have up-regulated Foxp3 expression, which is a transcription factor specifically expressed in CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells that programme its development and function<sup>(26)</sup>. Recently, CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells were found to play a pivotal role in the maintenance of tolerance in rodents and human subjects<sup>(27)</sup>. Additionally, many studies have provided compelling evidence that CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells can re-establish self-tolerance and prevent autoimmune diseases<sup>(10)</sup>. It should be noted that Wolf *et al.*<sup>(28)</sup> provided strong evidence that CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells are potent suppressors of anti-glomerular basement membrane GN in mice. It has also been reported that depletion of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells exacerbates the development of GN in NZB/W F1 mice<sup>(29)</sup>. In the present study, the suppressive effect of curcumin on IgG1 and IgG2a production was inhibited after the elimination of CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells. This result strongly indicates that delaying the disease pathogenesis in curcumin-treated mice is attributed to the direct or indirect effect of curcumin on CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells. However, the present study is limited to the relation between T<sub>reg</sub> cells and curcumin, which confirmed that the T<sub>reg</sub> cell has a major therapeutic role in LN of NZB/W F1 mice. Curcumin treatment could affect other immune cells, which might cause a cascade effect to the T<sub>reg</sub> cells' function. Though the present data strongly indicate that the immune-suppressive effect of curcumin is highly related to CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells in LN, these results imply a further need to assess the mechanism of action of curcumin on individual immune cells and to elucidate the effect of curcumin on CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells in LN.

### Conclusion

Overall, the results of the present study document the therapeutic effect of dietary curcumin in the attenuation of renal manifestations of LN in NZB/W lupus mice for the first time. Although it is still necessary to elucidate the specific immune-modulating signalling mechanism of curcumin in LN, the findings presented herein indicate that protective

effects of curcumin in LN may involve, at least in part, its interaction with CD4<sup>+</sup>CD25<sup>+</sup> T<sub>reg</sub> cells.

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### References

1. Swaak AJ, van den Brink HG, Smeenk RJ, *et al.* (1999) Systemic lupus erythematosus: clinical features in patients with a disease duration of over 10 years, first evaluation. *Rheumatology (Oxford, England)* **38**, 953–958.
2. Agrawal N, Chiang LK & Rifkin IR (2006) Lupus nephritis. *Semin Nephrol* **26**, 95–104.
3. Mason LJ & Berden JH (2008) Pathogenic factors for the development of lupus nephritis. *Lupus* **17**, 251–255.
4. Mortensen ES, Fenton KA & Rekvig OP (2008) Lupus nephritis: the central role of nucleosomes revealed. *Am J Pathol* **172**, 275–283.
5. Bagavant H & Fu SM (2009) Pathogenesis of kidney disease in systemic lupus erythematosus. *Curr Opin Rheumatol* **21**, 489–494.
6. Chan OT, Madaio MP & Shlomchik MJ (1999) The central and multiple roles of B cells in lupus pathogenesis. *Immunol Rev* **169**, 107–121.
7. Wofsy D & Seaman WE (1985) Successful treatment of autoimmunity in NZB/NZW F1 mice with monoclonal antibody to L3T4. *J Exp Med* **161**, 378–391.
8. Houssiau FA (2004) Management of lupus nephritis: an update. *J Am Soc Nephrol* **15**, 2694–2704.
9. Raimondi G, Turner MS, Thomson AW, *et al.* (2007) Naturally occurring regulatory T cells: recent insights in health and disease. *Crit Rev Immunol* **27**, 61–95.
10. Sakaguchi S, Ono M, Setoguchi R, *et al.* (2006) Foxp3<sup>+</sup> CD25<sup>+</sup> CD4<sup>+</sup> natural regulatory T cells in dominant self-tolerance and autoimmune disease. *Immunol Rev* **212**, 8–27.
11. Nishimura E, Sakihama T, Setoguchi R, *et al.* (2004) Induction of antigen-specific immunologic tolerance by *in vivo* and *in vitro* antigen-specific expansion of naturally arising Foxp3<sup>+</sup> CD25<sup>+</sup> CD4<sup>+</sup> regulatory T cells. *Int Immunol* **16**, 1189–1201.
12. Minami R, Sakai K, Miyamura T, *et al.* (2006) [The role of CD4<sup>+</sup> CD25<sup>+</sup> regulatory T cells in patients with rheumatoid arthritis]. *Nihon Rinsbo Meneki Gakkai Kaishi* **29**, 37–42.
13. Viglietta V, Baecher-Allan C, Weiner HL, *et al.* (2004) Loss of functional suppression by CD4<sup>+</sup> CD25<sup>+</sup> regulatory T cells in patients with multiple sclerosis. *J Exp Med* **199**, 971–979.
14. Alvarado-Sanchez B, Hernandez-Castro B, Portales-Perez D, *et al.* (2006) Regulatory T cells in patients with systemic lupus erythematosus. *J Autoimmun* **27**, 110–118.



15. Ammon HP & Wahl MA (1991) Pharmacology of *Curcuma longa*. *Planta Med* **57**, 1–7.
16. Natarajan C & Bright JJ (2002) Curcumin inhibits experimental allergic encephalomyelitis by blocking IL-12 signaling through Janus kinase-STAT pathway in T lymphocytes. *J Immunol* **168**, 6506–6513.
17. Sugimoto K, Hanai H, Tozawa K, *et al.* (2002) Curcumin prevents and ameliorates trinitrobenzene sulfonic acid-induced colitis in mice. *Gastroenterology* **123**, 1912–1922.
18. Khajehdehi P, Zanjanejad B, Aflaki E, *et al.* (2012) Oral supplementation of turmeric decreases proteinuria, hematuria, and systolic blood pressure in patients suffering from relapsing or refractory lupus nephritis: a randomized and placebo-controlled study. *J Ren Nutr* **22**, 50–57.
19. Foster MH (1999) Relevance of systemic lupus erythematosus nephritis animal models to human disease. *Semin Nephrol* **19**, 12–24.
20. Limtrakul P, Lipigorngoson S, Namwong O, *et al.* (1997) Inhibitory effect of dietary curcumin on skin carcinogenesis in mice. *Cancer Lett* **116**, 197–203.
21. Hori S, Nomura T & Sakaguchi S (2003) Control of regulatory T cell development by the transcription factor Foxp3. *Science* **299**, 1057–1061.
22. Szabo SJ, Kim ST, Costa GL, *et al.* (2000) A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* **100**, 655–669.
23. Yamashita M, Ukai-Tadenuma M, Miyamoto T, *et al.* (2004) Essential role of GATA3 for the maintenance of type 2 helper T (Th2) cytokine production and chromatin remodeling at the Th2 cytokine gene loci. *J Biol Chem* **279**, 26983–26990.
24. Ando DG, Sercarz EE & Hahn BH (1987) Mechanisms of T and B cell collaboration in the *in vitro* production of anti-DNA antibodies in the NZB/NZW F1 murine SLE model. *J Immunol* **138**, 3185–3190.
25. Stevens TL, Bossie A, Sanders VM, *et al.* (1988) Regulation of antibody isotype secretion by subsets of antigen-specific helper T cells. *Nature* **334**, 255–258.
26. Fontenot JD, Gavin MA & Rudensky AY (2003) Foxp3 programs the development and function of CD4<sup>+</sup> CD25<sup>+</sup> regulatory T cells. *Nature Immunol* **4**, 330–336.
27. Sakaguchi S, Sakaguchi N, Asano M, *et al.* (1995) Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* **155**, 1151–1164.
28. Wolf D, Hohegger K, Wolf AM, *et al.* (2005) CD4<sup>+</sup> CD25<sup>+</sup> regulatory T cells inhibit experimental anti-glomerular basement membrane glomerulonephritis in mice. *J Am Soc Nephrol* **16**, 1360–1370.
29. Hayashi T, Hasegawa K & Adachi C (2005) Elimination of CD4(+)CD25(+) T cell accelerates the development of glomerulonephritis during the preactive phase in autoimmune-prone female NZB × NZW F mice. *Int J Exp Pathol* **86**, 289–296.