(S)-armepavine from Chinese medicine improves experimental autoimmune crescentic glomerulonephritis

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Abstract

Objective. Intra-renal T cells and macrophages play a key pathogenic role in the development and progression of glomerular crescents. We aimed to establish (S)-armepavine [(S)-ARM], a major bioactive compound of a Chinese medicinal plant, Nelumbo nucifera, as a potential therapeutic agent in the treatment of autoimmune crescentic glomerulonephritis (ACGN).

Methods. A mouse ACGN model associated with T-cell dysregulation, was used to evaluate the therapeutic effects of (S)-ARM on the rapidly progressive glomerular disorder.

Results. The results showed that (S)-ARM administered in the established phase of ACGN is capable of dramatically decreasing glomerular crescents in the kidney and improving proteinuria and renal dysfunction. These effects were associated with greatly inhibited infiltration of T cells/macrophages and suppressed nuclear factor (NF)-κB activation in the kidney, lowered serum levels of autoantibodies and both serum and intra-renal levels of pro-inflammatory cytokines, suppressed T/B-cell activation and T-cell proliferation of the spleen, reduced glomerular immune deposits and apoptosis in both the spleen and kidney in (S)-ARM-treated ACGN mice, compared with the vehicle-treated (disease control) group of ACGN mice.

Conclusion. We demonstrated therapeutic effects of (S)-ARM on ACGN as a result of: (i) early systemic negative modulation of T/B cells; (ii) intra-renal regulation of combined NF-κB activation and mononuclear leucocytic infiltration, thereby preventing glomerular crescent formation; and (iii) protection from apoptosis in both the spleen and kidney.

Key word: Crescentic glomerulonephritis, Crescent, (S)-armepavine, Nelumbo nucifera, T cell, Macrophage, Nuclear factor-κB, Apoptosis.

Introduction

Autoimmune crescentic glomerulonephritis (ACGN) is an extremely progressive form of glomerulonephritis and is classified as a type of crescentic glomerulonephritis (rapidly progressive glomerulonephritis), in which extensive glomerular crescents consisting of a mixture of epithelial cells, macrophages and lymphocytes, are formed [1–4]. Of note, deletion of T cells and/or macrophages attenuates crescentic glomerulonephritis [2, 4–6], suggesting an essential role for mononuclear leucocytes, especially T cells and macrophages, in the pathogenesis of glomerular crescent formation. Unfortunately, the current therapy for crescentic glomerulonephritis, including ACGN, is still poor, and many patients shortly require long-term dialysis or renal transplantation [1].

Nelumbo nucifera, an eatable and Chinese medicinal plant has been used to treat diarrhoea, inflammation and haemostasis [7–10]. In a recent publication, we observed that treatment with (S)-armepavine [(S)-ARM], a major
bioactive compound of *N. nucifera*, is able to mitigate the progression of SLE in MRL/lpr mice, including attenuated mesangial cell hypercellularity and glomerular immune deposition [7]. This prompted us to further evaluate the effects of (S)-ARM on the diffuse formation of crescents of ACGN especially the pathogenic role of T cells and macrophages infiltrating locally in the kidney, which has been widely considered as a major mechanism responsible for widespread crescent glomerular crescent formation characteristic of crescentic glomerulonephritis [2, 3].

We treated the ACGN mice daily with (S)-ARM by oral gavage 2 weeks after induction of disease, which is basically a modified chronic graft vs host disease model, induced in C57BL/6 × DBA/J F1 hybrid mice by giving DBA/2J donor lymphocytes, with extensive glomerular crescent formation, sclerosis and interstitial inflammation, associated with abnormal ‘hyperactive’ T cells [11, 12], to verify the effectiveness of (S)-ARM on ACGN and determine the underlying relevant mechanisms. (S)-ARM administration effectively mitigated the evolution of ACGN by averting glomerular crescent formation and renal inflammation, involving a key mechanism of blocking intra-renal nuclear factor (NF)-κB activation and infiltration of T cells/macrophages, and inhibiting apoptosis in the kidney. The mode of action of (S)-ARM exerting its therapeutic effects on this ACGN model was addressed.

**Methods**

**Isolation of (S)-ARM from seeds of *N. nucifera***

(S)-ARM (C19H23O3N; MW 313) was performed by extraction of seeds of *N. nucifera* (1.2 kg) in ethanol as described previously [8]. Briefly, the water-soluble bioactive fraction was subjected to column chromatography in Diaion HP-20 with a water–methanol gradient. The factions of 75–100% methanol eluate were further purified by Sephadex LH-20 column repeatedly to generate (S)-ARM.

**ACGN model and (S)-ARM treatment**

ACGN was induced in 7- to 8-week-old female C57BL/6 × DBA/2J F1 hybrid mice by injecting them with DBA/2J donor lymphocytes, as described previously [11, 12]. Briefly, a cell suspension containing a mixture of donor cells from the thymus, spleen and lymph nodes (neck, axillary and inguinal regions) was injected intravenously three times at 4-day intervals. Eleven mice per group were used throughout the experiment, and were treated with (S)-ARM (8.5 mg/kg) or vehicle (corn oil) as disease control via oral gavage daily for totally 2 months, and the first dose was given at 2 weeks after the disease induction. Age-matched, without disease induction, C57BL/6 × DBA/2J F1 hybrid mice were used as normal control. All mice were sacrificed by cervical dislocation at Week 3 or 10 after disease induction. Spleen, renal cortical tissue, blood and urine samples were collected and stored appropriately until analysis. All animal experiments were performed with the approval of the Institutional Animal Care and Use Committee of The National Defense Medical Center, Taiwan and were consistent with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

**Clinical and pathological evaluation**

Collection and assay of blood and urine samples were performed as described previously [11]. Urine samples were collected in metabolic cages weekly and urinary levels of protein determined using a Pierce BCA protein assay kit (Perbio Science, Etten-Leur, The Netherlands), while serum samples were collected at Weeks 3 and 10, respectively, to measure blood urea nitrogen (BUN) and creatinine (Cr).

For histopathology, the tissues were fixed in 10% buffered formalin and embedded in paraffin. Sections (4 μm) were stained with haematoxylin and eosin. One hundred glomeruli were examined by light microscopy at the magnification of ×400 for the slide with at least two renal tissue sections each. Scoring of the severity of renal lesions was performed as described previously [12]. For scoring, 50 randomly selected glomeruli were examined. The proportion (percentage) was calculated for the following major three components: crescent formation, glomerular sclerosis and peri-glomerular inflammation.

**IF, immunohistochemistry and detection of apoptosis**

For the IC, MCP-1 and IL-6 detection, frozen renal tissues were cut, air-dried, fixed in acetone for 5 min at room temperature, and incubated with FITC-conjugated goat anti-mouse immunoglobulin G (IgG), C3 (Cappel, Durham, NC, USA) or goat anti-MCP-1 (Santa Cruz Biotechnology, Santa Cruz, CA), and rat anti-IL-6 (R&D Systems, Minneapolis, MN). Fifty glomeruli were examined on each slide and assigned values of staining intensity from 0 to >3. The total intensity score was calculated according to the following equation for each specimen: total intensity score = (% glomeruli intensity negative × 0) + (% glomeruli intensity trace intensity × 0.5) + (% glomeruli 1 + intensity × 1) + (% glomeruli 2 + intensity × 2) + (% glomeruli 3 + intensity × 3). The values ranged from 0 to a maximum of 300 [12].

For immunohistochemistry (IHC), methyl Carnoy’s solution or formalin-fixed, paraffin-embedded tissue sections (4 μm) were stained with rat anti-F4/80 (monocytes/macrophages; Serotec, Raleigh, NC, USA), or rabbit anti-pNF-κB p65 (Cell Signaling, Beverly, MA, USA) antibodies. To detect pan-T cells (CD3), Th cells (CD4) and monocytes/macrophages (CD11b), renal tissues were fixed in periodate–lysine paraformaldehyde as described previously [13], then incubated with biotin-conjugated anti-mouse CD3 (Serotec), CD4 (BioLegend, San Diego, CA, USA) or CD11b (BD Biosciences, San Jose, CA, USA) antibodies. Scoring of staining intensity for the glomerular (including peri-glomerular area) was performed as described previously [13].

For the detection of apoptosis, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling
(TUNEL) was employed. Formalin-fixed tissue sections were stained with ApopTag plus peroxidase in situ apoptosis detection kit (Chemicon, Temecula, CA, USA) according to the manufacturer’s instructions. For scoring, the spleen or cortical renal area (including glomerular and peri-glomerular area) examined by light microscopy at the magnification of ×400, as described previously [12].

For detection of serum ANA, indirect IF was performed according to the manufacturer’s instructions. The diluted sera were incubated on HEp-2 slides (Binding Site Ltd, Birmingham, UK), and then FITC-conjugated anti-mouse IgG antibody (Chemicon) was applied to the sections.

Flow cytometry

Splenocytes from the mice were treated with Tris-buffered ammonium chloride to eliminate erythrocytes, washed, resuspended in RPMI 1640 supplemented with 10% fetal calf serum, HEPES buffer, L-glutamine and penicillin/streptomycin (all from Gibco; Invitrogen, Carlsbad, CA, USA). The cells were stained with surface marker for T/B-cell activation or cell number count. FITC-conjugated anti-mouse CD3 (17A2), or CD19 (B cell, 1D3) antibodies and phycoerythrin (PE)-conjugated anti-mouse CD69 (H1.2F3) antibodies (all from BD Biosciences) were employed for the analysis of surface markers for T- or B-cell activation with FACSCalibur (BD Biosciences), as described previously [12]. For thymidine incorporation assay, splenocytes were cultured in triplicate in wells (5 × 10^5 cells in 200 μl/well) in 96-well flat-bottomed microtitre plates previously coated overnight at 4°C with 0.25 μg/ml of anti-mouse CD3 (145-2C11) antibodies (BD Biosciences). After 48 h, the cultures were pulsed with 1 μCi of 3H-methyl thymidine (Amersham Pharmacia Biotech, NJ, USA), harvested 16 h later, and the incorporated 3H-methyl thymidine measured using a TopCount (Packard; PerkinElmer, Boston, MA, USA) as described previously [12].

For concanavalin A (Con-A) assay, to evaluate the immunosuppressive effects of (S)-ARM on splenocytes from C57/DBA 2J F1 mice, the cells were incubated with various concentrations of (S)-ARM (3.12, 6.25 and 12.5 μM) 30 min before Con-A stimulation for 48 h, as described previously with some modification [7]. The dose of (S)-ARM showed no direct cytotoxicity, as described previously [7, 8]. Treatment with the vehicle (0.1% dimethylsulphoxide) did not affect the resting or the stimulated state.

ELISA

Serum levels of MCP-1, INF-γ and IL-4 were measured using commercial ELISA kits (BD Biosciences) according to the manufacturer’s instructions. Anti-dsDNA antibody was measured using an anti-mouse dsDNA ELISA kit (Alpha Diagnostic, San Antonio, TX, USA). In all ELISAs, the absorbance at 450 nm was measured using an ELISA plate reader (Bio-Tek, Winooski, MA, USA).

Statistical analysis

Values are presented as the mean (S.E.M.). Comparison between two groups was performed using Student’s t-test. P < 0.05 was considered statistically significant.

Results

(S)-ARM improves proteinuria, renal function and pathology

ACGN mice were treated daily with (S)-ARM 2 weeks after disease induction for totally 2 months, and showed significantly improved proteinuria at Week 6, compared with vehicle (corn oil)-treated ACGN (disease control) mice (Fig. 1A). This effect was found to persist until Week 10 when the mice were sacrificed. All of the ACGN mice survived until sacrificed, although some of them showed moderate or severe ascites at the endpoint of the study. Likewise, a dramatic improvement in renal function was noted in the (S)-ARM-treated ACGN mice, as demonstrated by significantly lower serum levels of BUN (Fig. 1B; P < 0.05) and Cr (Fig. 1C; P < 0.05) seen in the (S)-ARM-treated mice than in vehicle-treated ACGN (disease control) mice at Week 10 after the induction of disease. There was no significant difference in the serum levels of BUN or Cr between the (S)-ARM- and vehicle-treated ACGN (disease control) mice at Week 3.

Histopathologically, the vehicle-treated ACGN mice revealed extensive glomerular crescent formation, intense interstitial inflammation and glomerulosclerosis at Week 10 (Fig. 1D and E; each P < 0.005). In contrast, these progressive renal lesions were greatly reduced in ACGN mice that were treated with (S)-ARM (Fig. 1D and E; each P < 0.005). There were no detectable histopathological changes in the vehicle- or (S)-ARM-treated ACGN mice at Week 3.

Importantly, as early as Week 3, vehicle-treated ACGN mice developed diffuse IgG deposition in the glomerulus, but significant inhibition of this effect was observed in (S)-ARM-treated ACGN mice (Fig. 2A–C and M; P < 0.01). At Week 10, again the (S)-ARM-treated ACGN mice showed significantly less IgG deposition than vehicle-treated ACGN mice (Fig. 2H, I and N; P < 0.005). Similarly, (S)-ARM-treated ACGN mice also showed significant inhibitory effects on glomerular C3 deposition at Week 10 (Fig. 2J–L and N; P < 0.005), although there was no significant difference in glomerular C3 deposits between the two groups at Week 3 (Fig. 2D–F and M).

(S)-ARM reduces serum levels of autoantibodies

As autoantibodies play a major pathogenic role in the development of SLE [14, 15], we measured serum levels of anti-dsDNA and ANA antibodies at Weeks 3 and 10, respectively, after the induction of ACGN. As shown in Fig. 3A, serum levels of anti-dsDNA autoantibody were significantly lower as early as Week 3 in the (S)-ARM-treated ACGN mice (P < 0.01) than in vehicle-treated ACGN (disease control) mice, as demonstrated by ELISA, and this effect lasted until Week 10 when the mice were sacrificed (P < 0.005). Furthermore,
serum ANA antibody levels were significantly decreased in (S)-ARM-treated ACGN mice compared with vehicle-treated (disease control) ACGN mice, as demonstrated by IF assay (Fig. 3B).

(S)-ARM can suppress T/B-cell activity in the spleen Abnormal T- and B-cell interaction can result in a graft vs host reaction [16, 17], the basic mechanism for induction of the ACGN model [12]. We demonstrated that (S)-ARM inhibited T-cell proliferation in MRL/lpr mice [8] via blocking of the membrane-proximal effectors (such as Itk and PLCr in a PI-3k-dependent manner) as the action mechanism responsible for suppressing T-cell activity [7]. We further tested whether (S)-ARM administration could improve the renal lesions, especially glomerular crescent formation during the evolution of the ACGN model by negatively regulating (i) T- and/or B-cell activation by performing flow cytometry; and (ii) T-cell proliferation by thymidine incorporation test in splenocytes, as follows.

Inhibition of T/B-cell activation. As shown in Fig. 4A, the CD3^+CD69^+ cells (activated T cells) from the spleen were significantly increased in vehicle-treated ACGN (disease control) mice at both Weeks 3 and 10 compared with normal control mice (each \( P < 0.005 \)). Obviously, this effect was abolished by the treatment with (S)-ARM of ACGN mice compared with vehicle-treated ACGN (disease control) mice (each \( P < 0.005 \)). Similarly, CD19^+CD69^+ cells (activated B cells) were significantly reduced in (S)-ARM-treated ACGN mice to the levels seen in normal control mice at Weeks 3 and 10 (Fig. 4B; each \( P < 0.01 \)). Furthermore, the total numbers of CD3 and CD19 in the spleen were significantly decreased in (S)-ARM-treated ACGN mice compared with vehicle-treated ACGN (disease control) mice at Week 3 (CD3: 32 ± 6 × 10^6 vs 51 ± 8 × 10^6; CD19: 58 ± 12 × 10^6 vs 107 ± 25 × 10^6) (each \( P < 0.05 \)) and Week 10 (CD3: 39 ± 2 × 10^6 vs 72 ± 15 × 10^6; CD19: 60 ± 22 × 10^6 vs 140 ± 30 × 10^6) (each \( P < 0.05 \)).

Inhibition of T-cell proliferation. As shown in Fig. 4C, compared with vehicle-treated ACGN (disease control) mice, (S)-ARM-treated ACGN mice showed significant suppression of spleen T-cell proliferation to the level seen in normal control mice early at Weeks 3 and 10 (\( P < 0.005 \), Week 3; \( P < 0.05 \), Week 10). Besides, we further evaluated the immunosuppressive effects of (S)-ARM on splenocytes from C57/DBA 2J F1 mice. Although significantly enhanced proliferation was observed in Con-A-stimulated splenocytes (460 752 ± 10 268 c.p.m.) compared with those without Con-A...
Fig. 2 (S)-ARM reduces glomerular immune deposits. (A–F) Week 3; (G–L) Week 10. (A–C and G–I) IgG; (D–F and J–L) C3. Original magnification, ×400. (M, N) Staining intensity score. Each bar represents the mean (S.E.M.) for a group of 11 mice treated with (S)-ARM (hatched bars), vehicle (solid bars) or normal control (open bars). **P < 0.01 and ***P < 0.005. NS: no significant difference.

Fig. 3 (S)-ARM reduces autoantibody production. (A) Anti-dsDNA levels by ELISA. (B) ANA indirect IF using HEp-2 slides. Original magnification, ×400. Each bar represents the mean (S.E.M.) for a group of 11 mice treated with (S)-ARM (hatched bars), vehicle (solid bars) or normal control (open bars). **P < 0.01 and ***P < 0.005.
cytokine production, we measured serum levels of these proteins in the mice. As early as Week 3, serum levels of MCP-1 of (S)-ARM-treated ACGN mice were significantly lower than those of vehicle-treated ACGN (disease control) mice (Fig. 5A; \( P < 0.05 \)), although there was no significant difference in the levels of INF-\( \gamma \) and IL-4 between the two groups (Fig. 5B and C). At Week 10, however, significantly lower serum levels of all these cytokines were seen in (S)-ARM-treated ACGN mice than vehicle-treated ACGN (disease control) mice (Fig. 5A–C; each \( P < 0.05 \)).

(S)-ARM mitigates the intra-renal infiltration of T cells and macrophages

Intra-renal infiltration of T cells [6, 24] and/or monocytes/macrophages [2, 6] plays a pivotal pathogenic role in the development of glomerular crescents of crescentic glomerulonephritis, including ACGN. Lan et al. [4] proposed that macrophage accumulation within crescents plays an important role in the progression of epithelial-dominated early cellular crescents to macrophage-dominated advanced and fibrocellular crescents. In our work, we assessed whether intra-renal infiltration of mononuclear leucocytes was suppressed by (S)-ARM treatment. At Week 10, IHC showed a diffuse infiltration of T cells (CD3\(^+\) or CD4\(^+\)) and monocytes/macrophages (CD11b\(^+\) or F4/80\(^+\); Fig. 6B and D) in the peri-glomerular region of the renal interstitium in vehicle-treated ACGN mice (disease control). In contrast, this effect was dramatically reduced by the treatment with (S)-ARM (each \( P < 0.01 \)). Only a few or no mononuclear cells were noted in either vehicle-treated ACGN (disease control) or (S)-ARM-treated ACGN (Fig. 6A and C) early at Week 3.

(S)-ARM can inhibit the production of intra-renal pro-inflammatory cytokines by negatively modulating NF-\( \kappa \)B activation

Renal expression of pro-inflammatory cytokines, such as MCP-1 and IL-6, has been demonstrated to play a pivotal role in the pathogenesis of crescent glomerulonephritis [2, 13, 25–27]. Activation of NF-\( \kappa \)B results in expression of genes that encode these proteins [28, 29]. As shown by IHC in Fig. 7B and D, first, (S)-ARM-treated ACGN mice showed significantly reduced renal protein expression levels of MCP-1 and IL-6, compared with the vehicle-treated ACGN (disease control) mice (each \( P < 0.005 \)) at Week 10. Next, NF-\( \kappa \)B p65 activation was significantly inhibited in the kidney of (S)-ARM-treated ACGN mice, compared with the vehicle-treated ACGN (disease control) mice (Fig. 7B and D), as demonstrated by IHC. There was no significant difference in protein expression of MCP-1, IL-6 and NF-\( \kappa \)B p65 in either vehicle-treated ACGN (disease control) mice or (S)-ARM-treated ACGN mice (Fig. 7A and C) early at Week 3.

(S)-ARM can prevent apoptosis in both the spleen and kidney

Abnormal regulation of apoptosis has been implicated in the pathogenesis of various glomerular disorders [30–34].

(S)-ARM can reduce serum levels of pro-inflammatory cytokines

MCP-1 [18, 19], IL-4 [20, 21] and INF-\( \gamma \) [22, 23] have been considered to play a critical role in the pathogenesis of crescentic glomerulonephritis. To determine whether (S)-ARM treatment affected systemic pro-inflammatory cytokine production, we measured serum levels of these proteins in the mice. As early as Week 3, serum levels of MCP-1 of (S)-ARM-treated ACGN mice were significantly lower than those of vehicle-treated ACGN (disease control) mice (Fig. 5A; \( P < 0.05 \)), although there was no significant difference in the levels of INF-\( \gamma \) and IL-4 between the two groups (Fig. 5B and C). At Week 10, however, significantly lower serum levels of all these cytokines were seen in (S)-ARM-treated ACGN mice than vehicle-treated ACGN (disease control) mice (Fig. 5A–C; each \( P < 0.05 \)).

(S)-ARM averts crescent formation
We evaluated apoptosis of the spleen and kidney in the mice by the TUNEL assay. As early as Week 3, significant suppression of apoptosis in the spleen was seen in (S)-ARM-treated ACGN mice, compared with vehicle-treated ACGN (disease control) mice (Fig. 8A and C). Similarly, only very few apoptotic cells were identified in (S)-ARM-treated ACGN mice at Week 10 (Fig. 8B and D), although suppression was frequently identified in the spleen of vehicle-treated ACGN (disease control) mice. In parallel, significant suppression of apoptosis was observed in the glomeruli and some renal tubules of (S)-ARM-treated ACGN mice, compared with vehicle-treated ACGN (disease control) mice at this point (Fig. 8B and D). No detectable apoptosis in the glomeruli was identified early at Week 3 in vehicle- or (S)-ARM-treated ACGN mice, although few apoptotic figures were seen in their renal tubules (Fig. 8A and C).

### Discussion

Our study demonstrates dramatic therapeutic effects of (S)-ARM, extracted from *N. nucifera* (a Chinese medicinal plant) on an ACGN mouse model, by greatly reducing glomerular crescent formation and T-cell/macrophage infiltration in the kidney. As schematic in Fig. 9, we propose the following mechanisms potentially responsible for its effectiveness on the ACGN model: (i) early systemic modulation of T-cell activation/proliferation and B-cell activation; (ii) intra-renal regulation of combined NF-κB activation and mononuclear leucocytic infiltration, thereby preventing glomerular crescent formation; and (iii) protection against splenic and intra-renal apoptosis.

First, it is well known that abnormal T-cell function plays a pathogenic role in SLE [35–37]. We propose that (S)-ARM systemically modulates T- and B-cell activation and/or proliferation as a key mechanism responsible for the effects of (S)-ARM on the ACGN model. We have previously demonstrated that (S)-ARM has systemic effects on negatively regulating T-cell function [7] and lymphokine production [8]. In the present study, we showed that even at early stage (Week 3) of the disease model, (S)-ARM administration was shown to significantly inhibit T/B-cell activation and T-cell proliferation in the treated ACGN mice. This effect was closely associated with significantly reduced serum autoantibody levels and glomerular immune deposits. Furthermore, it should be noted that IL-4 [20, 21] and INF-γ [22, 23] contribute to the pathogenesis of human lupus nephritis. We showed that (S)-ARM administration also greatly lowered the serum levels of both IL-4 and IFN-γ at Week 10. This can in part explain the favourable effects of (S)-ARM on the particular renal lesions with diffuse crescents in the ACGN mice. On the other hand, detection of a broader detection of serum levels of pro-inflammatory (e.g. TNF-α, IL-6 and IL-1) and maybe anti-inflammatory cytokines (such as IL-10) would also be helpful in evaluating the effectiveness of (S)-ARM treatment.

As for the second potential mechanism—combined regulation of both NF-κB activation and mononuclear leucocytic infiltration, it is known that intra-renal expression of NF-κB-dependent pro-inflammatory cytokines, including MCP-1 and IL-6, plays a critical pathogenic role in the development and progression of crescentic glomerulonephritis [2, 13, 25–27]. Blocking of NF-κB activation has been reported to inhibit crescentic glomerulonephritis in a rat model, confirming a critical pathogenic role for the NF-κB-dependent inflammatory pathway in progressive renal injury [38]. Deletion of T cells and/or macrophages has been shown to mitigate crescentic glomerulonephritis [2, 4, 6], suggesting a crucial pathogenic role of mononuclear leukocytes in the development of glomerular crescents of crescentic glomerulonephritis. At the glomerulus level, it is generally accepted that a glomerular crescent consists of proliferation of the parietal epithelial cells and mononuclear leucocyte infiltrates [1, 3]. Importantly, intra-renal infiltration of T cells [6, 24] and/or monocytes/macrophages [2, 6] has been closely linked to the formation and progression of glomerular crescents. Furthermore, macrophage accumulation within glomerular crescents mediates the progression of epithelial-dominated early cellular crescents to macrophage-dominated advanced and fibrocellular crescents [4]. Based on our data, (S)-ARM administration obviously resulted in: (i) significant decrease in serum MCP-1 levels as early as Week 3; and (ii) nearly total absence of periglomerular interstitial mononuclear leucocyte infiltration. These effects operating locally in the kidney to suppress mononuclear leucocyte infiltrations could serve as an
immediate and crucial mechanism for the beneficial therapeutic effects of (S)-ARM on ACGN (Fig. 9). Thus, blockade of NF-κB activation in both glomeruli and interstitial tissues could be a key mechanism by which (S)-ARM administration ameliorates immune-mediated renal injury in this ACGN model. The third possible mechanism is that (S)-ARM prevents apoptosis in the spleen and kidney. High apoptotic rates...
are identified in severe glomerular lesions of glomerulonephritis patients [31, 39, 40]. Obviously, our data showed that prevention of apoptosis in both the spleen and kidney, including glomerular and tubulointerstitial compartments, was closely associated with lower histopathological severity of the progressive renal lesion in the (S)-ARM-treated ACGN mice (Fig. 9). Although relatively high doses of armepavine oxalate, which bears very high similarity to (S)-ARM, have been shown to induce apoptosis in a leukaemia cell line [41], we observed no such a side effect at the dose we employed throughout this experiment in both the lymphoid tissues (as shown in the spleen) and kidney. Besides, reduced apoptosis in lymphoid tissues can result in an autoimmune status in

Fig. 7 (S)-ARM inhibits renal inflammatory cytokine IL-6, MCP-1 and NF-κB p65 expression. (A) Week 3. (B) Week 10. Original magnification, ×400. (C, D) Scoring of intensity score or positive cells in kidney [including glomerular and peri-glomerular (interstitial) area]. Each bar represents the mean (S.E.M.) for a group of 11 mice treated with (S)-ARM (hatched bars), vehicle (solid bars) or normal control (open bars). *P < 0.05 and ***P < 0.005. g.s.: glomerular section; NS: no significant difference.
FIG. 8 (S)-ARM prevents apoptosis in the spleen and kidney. (A) Week 3. (B) Week 10. Original magnification, ×400. (C, D) Scoring of positive cells in spleen and kidney [including glomerular and peri-glomerular (interstitial) area]. Each bar represents the mean (S.E.M.) for a group of 11 mice treated with (S)-ARM (hatched bars), vehicle (solid bars) or normal control (open bars). *P < 0.05, **P < 0.01 and ***P < 0.005. g.s.: glomerular section; NS: no significant difference. Arrows indicate the positively stained cells.

FIG. 9 Schematic diagram of the potential mechanisms responsible for the effectiveness of (S)-ARM in averting glomerular crescents. It is hypothesized that (S)-ARM inhibits: (i) systemic T/B-cell activation; (ii) local intra-renal T-cell/macrophage infiltration; (iii) combined intra-renal NF-κB-dependent pro-inflammatory cytokine production and mononuclear leucocytic infiltration; and (iv) intra-renal apoptosis. Tu: tubule.
lupus-prone MRL/lpr mice as a result of prolonged survival and proliferation of T cells [42]. In contrast, apoptosis may amplify autoantibody production of SLE due to increased intrinsic self-antigens (such as apoptotic bodies) [43, 44]. In this regard, we demonstrated that the administration of (S)-ARM greatly reduced apoptosis in both the kidney and lymphoid tissues (as represented by the spleen), and in part account for its beneficial effects on autoimmune rapid progressive glomerular disorders.

Besides, we demonstrated that the ACGN mouse model used in the current study involves elevated inducible nitric oxide synthase expression in both mRNA and protein in renal tissues in a recent publication [13].

In our previous studies, we demonstrated that (S)-ARM (i) has the ability to inhibit only activated T cells without affecting resting T cells in primary human peripheral blood mononuclear cells [7]; and (ii) showed no significant side effects in a lupus nephritis model of MRL/lpr mice [8]. For the time being, we and others [7] are actively working on preclinical assessments for further clinical trials.

In conclusion, our data show that administration of (S)-ARM effectively averts intense glomerular crescent formation and renal inflammation, thereby in part mitigating the evolution of ACGN. We provide evidence that hindering T-cell and macrophage infiltration locally in the kidney via negatively modulating the NF-κB-mediated inflammatory pathway associated with systemic modulation of T-cell activation/proliferation and B-cell activation might be the key mechanism responsible for the favourable therapeutic effects of (S)-ARM on ACGN. Prevention of apoptosis and/or free radicals in the spleen and kidney by (S)-ARM treatment is likely another important mode of action of (S)-ARM, a chemical extracted from N. nucifera, on this kind of rapidly progressive glomerular disorder.

**Rheumatology key messages**

- (S)-ARM averts glomerular crescent formation and renal inflammation.
- Responsible mechanism involves a negative regulation of a T/B cell and NF-κB-dependent pathway.

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