Abstract: In this study, we first analyzed the expression level of fractalkine (FKN) in the serum of patients with lupus nephritis (LN) and the distribution of peripheral blood Treg cells, and explored FKN and Treg cells, systemic lupus erythematosus disease activity index 2000 (SLEDAI-2K) correlation. Subsequently, we explored the specific role of FKN in tubule interstitial lesions (TILs) and regulatory T (Treg) cells/T helper (Th) 17 cell balance in lupus model mice. Treated with an anti-FKN antibody (aFKN), recombinant FKN (rFKN), or an isotype antibody (IgG) in MRL/MpJ-Faslpr/J and C57BL/6 mice, and then detected TIL level and forkhead box p3 (Foxp3), IL-10, IL-17 and IL-6 expression levels in the kidney and spleen in the proportion of Treg and Th17 cells. Finally, then use aFKN, rFKN, or IgG to intervene in polarized Tregs with IL-6, TGF-β, IL-23, anti-interferon, and Th17 cells with anti-IL-4 after transforming to transform growth factor (TGF)-β and interleukin (IL)-2 in isolated mouse spleen lymphocytes. The results showed that the expression level of FKN was positively correlated with SLEDAI-2K and negatively correlated with the distribution of Treg cells. After treatment with aFKN in lupus model mice, kidney damage was delayed, TIL formation was reduced, Foxp3 and IL-10 levels were up-regulated, IL-17 and IL-6 levels were down-regulated in renal tissues, Th17 cell subsets and Treg cell subsets were reduced The increase is in the spleen, and rFKN treatment has the opposite effect in mouse. In addition, after interfering with polarized cells by aFKN, it was found that IL-17 and IL-6 expression levels were down-regulated in Th17 cells, Foxp3 and IL-10 levels in Tregs were up-regulated, and rFKN treatment had the opposite effect in vitro. These results indicate that FKN participates in and promotes SLE target organ damage including: secretion of inflammatory factors and renal TIL, and most importantly, these effects might have been due to modification of the Treg/Th17 cell balance.

Keywords: Lupus nephritis, fractalkine, Treg/Th17 cells, tubulointerstitial lesions, murine model

Introduction

Systemic lupus erythematosus (SLE) is a complex multi-system autoimmune disease characterized by imbalance of immune tolerance, excessive proliferation of T lymphocytes, and inflammation of multiple organ tissues, which mediates excessive B cell activation and large production of autoantibodies. Importantly, under such conditions, the number and function of regulatory T cells (Tregs) and T helper (Th) 17 cells become imbalanced. Lupus nephritis (LN) is one of the most common and serious complications in patients with SLE, with associated pathologic changes in LN including a spectrum of glomerular and tubular edema, atrophy, interstitial inflammation, and/or fibrosis. Approximately 60% of patients with LN and symptoms of chronic kidney disease exhibit interstitial inflammation or fibrosis [1].

Fractalkine (FKN) is a novel membrane-bound chemokine comprising 373 amino acids and mainly expressed in endothelial cells. FKN exhibits chemotactic activity for macrophages, epithelial cells, and T lymphocytes and can mediate inflammatory damage through the action of adhesion molecules while participat-
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Lymphocyte proliferation plays a special function in promotion of renal tissue damage in human and experimental LN. Earlier immunological reports revealed new subsets of T cells, including Tregs, Th17 cells, and T follicular helper cells [8]. Maintenance of immune homeostasis and tolerance *in vivo* is beneficial to generation of a moderate immune response and regulation of balance in the number of T cell subsets. Tregs and Th17 cells exhibit completely different functions in immune responses, and the balance between these cells is critical for immune homeostasis and tolerance, with altered balance associated with LN pathogenesis in both humans and mice. Th17 cells are closely related to autoimmune diseases, chronic infections, and tumors by promoting secretion of interleukin (IL)-17A, IL-17F, RAR-related orphan receptor-yt, IL-22, and other cytokines to drive inflammation *in vivo* [9, 10]. Tregs play significant roles in inducing immune tolerance and regulating immune balance, mainly through the secretion of anti-inflammatory factors, including forkhead box p3 (Foxp3), IL-10, and transforming growth factor (TGF)-β [11]. Because depletion of naturally occurring Tregs leads to excessive proliferation of effector T cells, they play a crucial role in immune tolerance and regulation of immune balance [12, 13]. Our previous study [14] found that decreased and increased proportions of Tregs and Th17 cells, respectively, positively correlated with disease severity in LN. Additionally, previous studies reported that the number of Tregs decreases and that of Th17 cells increases in patients with SLE according to flow cytometry [15, 16]. These findings suggest that the pathogenesis of autoimmune diseases often arise in conflicting ways involving Tregs and Th17 cells [17, 18]; however, no studies have been conducted to determine an association with Treg/Th17 cell imbalance and FKN expression in LN. We hypothesized that the onset of LN patients might be due to increased levels of FKN that further promote proinflammatory T cell responses by increasing the number of Th17 cells and inhibiting the production of Tregs.

In our previous studies, we examined whether FKN stimulates tubulointerstitial fibrogenesis in MRL/lpr mice and determined whether FKN mediates the inflammatory process in lymphocytes [19]. In the present study, we first investigated the percentage of Treg cells in peripheral blood and serum FKN levels in SLE patients and healthy controls and assessed the relationship between FKN and Treg cells amount in SLE disease activity. Disease activity index 2000 (SLEDAI-2K) was used to evaluate SLE disease activity. Further explore the role of FKN in the altered subset of Tregs and Th17 cells, the production of IL-10, IL-17, and IL-6 *in vivo* and *in vitro*, which affect the formation of renal tubulo-interstitial fibrogenesis.

**Materials and methods**

**Antibodies and reagents**

The following reagents were purchased from R&D Systems (Minnesota, MN, USA): rat IgG2A isotype control (#CA02315081), recombinant mouse CX3CL1/FKN chemokine domain (#458-MF), and a mouse CX3CL1/FKN chemokine domain antibody (#EMX0217061). Human FKN ELISA kit (#DCX310) were purchased from R&D Systems (Minnesota, MN, USA). Antibodies against IL-6 (#18092), Foxp3 (sc-53876) and FKN (#F7059) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-IL-17 (ab79056) was purchased from Abcam (Shanghai, China), anti-IL-10 (#DF6894) was purchased from Affinity Biosciences (Changzhou, China), and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; #18AF0403) was purchased from ZSGB Biotechnology (Beijing, China).

**Clinical materials and design**

This study was approved by the Affiliated Hospital Ethics Committee of Youjiang Medical University for Nationalities (Baise, Guangxi, China). Written informed consent was obtained from all patients. All participants were initially
Table 1. Demographics, clinical and laboratory characteristics of SLE patients and healthy controls

<table>
<thead>
<tr>
<th>Variables</th>
<th>Health controls (n=11)</th>
<th>Inactive SLE (n=18)</th>
<th>Active SLE (n=13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at onset (years)</td>
<td>29 (28-46)</td>
<td>35 (29-40)</td>
<td>43 (38-49)</td>
</tr>
<tr>
<td>Sex (female:male ratio)</td>
<td>9:2</td>
<td>17:1</td>
<td>12:1</td>
</tr>
<tr>
<td>Disease duration (years)</td>
<td>NA</td>
<td>7.5 (5.6-8.5)</td>
<td>6.5 (5.7-7.9)</td>
</tr>
<tr>
<td>Anti-dsDNA (U/mL)</td>
<td>4.9 (4.1-6.1)</td>
<td>12.8 (8.6-27.7)</td>
<td>16.2 (13-49.7)</td>
</tr>
<tr>
<td>Anti-Sm (U/mL)</td>
<td>1 (0.88-1.2)</td>
<td>1.9 (1.6-2.2)</td>
<td>2.3 (2.3-3.1)</td>
</tr>
<tr>
<td>Serum C3 (g/L)</td>
<td>1.1 (0.98-1.5)</td>
<td>0.79 (0.73-0.94)</td>
<td>0.67 (0.61-0.68)</td>
</tr>
<tr>
<td>Serum C4 (g/L)</td>
<td>0.28 (0.19-0.33)</td>
<td>0.11 (0.10-0.13)</td>
<td>0.08 (0.07-0.09)</td>
</tr>
<tr>
<td>Baseline SLEDAI-2K score</td>
<td>NA</td>
<td>6 (5-7)</td>
<td>18 (13-21)</td>
</tr>
</tbody>
</table>

Medians and interquartile ranges (25%-75% quantile) are shown, where applicable. NA: not available. *Compared to healthy controls, P < 0.01. **Compared to inactive SLE, P < 0.05.

diagnosed with SLE (n=31) from July 2017 to August 2018 at the Affiliated Hospital of Youjiang Medical University for Nationalities, and healthy volunteer controls (n=11) were recruited over the same period. Healthy volunteer control serum samples were obtained from age- and sex-matched subjects with no known autoimmune conditions. The SLE patients fulfilled the international criteria on their disease activity, patients were divided into two groups according to their SLEDAI-2K scores: (1) inactive SLE with scores of < 10, and (2) active SLE with scores of ≥ 10 (Table 1). Any of the following clinical symptoms were considered a sign of active disease: polyarthritis, inflammatory skin symptoms, serositis, and clinical signs of active central nervous system or kidney manifestations. In addition to at least one of the above symptoms, clinically active patients also showed increased erythrocyte sedimentation rates and/or fever. Blood samples from all participants were collected for enzyme-linked immunosorbent assay (ELISA) and flow cytometry (FC) analysis. Blood samples were collected into serum separator tubes, allowed to clot for 30 min before centrifugation at 2,000×g for 20 min, separated into aliquots, and stored at -80°C for analysis.

Animals and experimental protocol

Female MRL/lpr and C57BL/6 mice were purchased from Better Biotechnology Co., Ltd. (Shandong, China) and housed in an environment between 22°C and 25°C and 40% and 60% relative humidity in a pathogen-free facility in the Animal Research Institute of Youjiang Medical University for Nationalities (Guangxi, China). All experimental mice were given free access to water and food and were exposed to a 12/12-h light/dark cycle. All mouse-related procedures were approved by the Committee of Animal Care and Use of Youjiang Medical University for Nationalities and in accordance with National Institutes of Health guidelines. All mice underwent intraperitoneal (i.p.) injections at 12-weeks old after randomization and were divided into eight groups (n=5 mice/group): 1) normal control (NC), C57BL/6 mice receiving 1 mL of normal saline daily; 2) LN group, MRL/lpr mice receiving 1 mL of normal saline daily; 3) NC + IgG group, C57BL/6 mice receiving 5 µg/mL IgG daily; 4) LN + IgG group, MRL/lpr mice receiving 5 µg/mL IgG daily; 5) NC + recombinant FKN (rFKN) group, C57BL/6 mice receiving 100 ng/mL rFKN daily; 6) LN + rFKN group, MRL/lpr mice receiving 100 ng/mL rFKN daily; 7) NC + anti-FKN antibody (aFKN) group, C57BL/6 mice receiving 5 µg/mL aFKN daily; and 8) LN + aFKN group, MRL/lpr mice receiving 5 µg/mL aFKN daily.

After 7 days of treatment with the antibodies, blood samples from female mice were collected by tail or retro-orbital puncture (using heparinized glass capillary tubes; EDTAK2; BD Medical Equipment Co., Ltd., Suzhou, China) under anesthesia. The spleen and kidney were detached under sterile conditions, and parts of the kidneys were initially stored at -80°C until use for determination of protein and RNA levels. Peripheral blood was collected for detection of blood urea nitrogen (BUN) and creatinine (Cr) levels to assess renal function. The details for these assays were described previously [19].

Cell isolation, culture, stimulation, and T cell polarization

To obtain a single-cell suspension from the spleen, organs were gently ground with a
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syringe tail and filtered through a 70-μm nylon mesh filter (LOT: 133034; Sigma-Aldrich, St. Louis, MO, USA). A mouse spleen lymphocyte isolation kit (#20171221; Solarbio, Beijing, China) was used according manufacturer instructions, and the lymphocyte-enriched cell suspension was aspirated from the Percoll interface. Red blood cells were lysed with ammonium chloride then washed with phosphate-buffered saline (PBS) three times and resuspended in Roswell Park Memorial Institute (RPMI)-1640 medium containing 10% fetal bovine serum.

The total CD4+ -enriched (CD4+ T cell isolation kit; Miltenyi, Bergisch Gladbach, Germany) fraction of mouse splenic lymphocytes were activated with plate-bound anti-CD3 (10 ng/mL; Dakewe Biotech, Shenzhen, China) and soluble anti-CD28 (1 mg/mL; Dakewe Biotech) for activation and polarization experiments. Cells were cultured in complete RPMI-1640 medium under Th17-polarizing conditions, which included IL-6 (50 ng/mL; Peprotech, Rocky Hill, NJ, USA), TGF-β (1 ng/mL; Peprotech), IL-23 (20 ng/mL; Peprotech), anti-interferon (IFN)γ (3 mg/mL; Dakewe Biotech), and anti-IL-4 (3 mg/mL; Dakewe Biotech), or in the absence of exogenous cytokines (Th0). Polarizations lasted 72 h unless otherwise noted. Foxp3 and IL-17 levels were detected by western blot and quantitative reverse transcription polymerase chain reaction (qRT-PCR).

In vitro experimental protocol

CD4+ T lymphocytes from the spleens of C57BL/6 (NC) and MRL/lpr mice (LN) were selected as controls, polarized into Tregs, and divided into eight groups: 1) TregNC, splenic CD4+ T cells from C57BL/6 mice (untreated); 2) TregLN, splenic CD4+ T cells from MRL/lpr mice (untreated); 3) TregNC + IgG, cells of the TregNC group treated with 1.5 μg/mL IgG; 4) TregLN + IgG, cells of the TregLN group treated with 1.5 μg/mL IgG; 5) TregNC + rFKN, cells of the TregNC group treated with 10 ng/mL rFKN; 6) TregLN + rFKN, cells of the TregLN group treated with 10 ng/mL rFKN; 7) TregNC + aFKN, cells of the TregNC group treated with 1.5 μg/mL aFKN; and 8) TregLN + aFKN, cells of the TregLN group treated with 1.5 μg/mL aFKN.

CD4+ T lymphocytes from spleens were polarized into Th17 cells and then divided into the following eight groups: 1) Th17NC, splenic CD4+ T cells from C57BL/6 mice (untreated); 2) Th17LN, splenic CD4+ T cells from MRL/lpr mice (untreated); 3) Th17NC + IgG, cells of the Th17NC group treated with 1.5 μg/mL IgG; 4) Th17LN + IgG, cells of the Th17LN group treated with 1.5 μg/mL IgG; (5) Th17NC + rFKN, cells of the Th17NC group treated with 10 ng/mL rFKN; 6) Th17LN + rFKN, cells of the Th17LN group treated with 10 ng/mL rFKN; 7) Th17NC + aFKN, cells of the Th17NC group treated with 1.5 μg/mL aFKN; and 8) Th17LN + aFKN, cells of the Th17LN group treated with 1.5 μg/mL aFKN.

Enzyme-linked immunosorbent assay (ELISA)

After anesthetizing the mice at the end of the experimental procedure, peripheral blood of mice was collected from the retro-orbital plexus using heparinized glass capillary tubes (EDTAK2; BD Medical Equipment Co.). Serum was collected by centrifugation at 1000 g for 15 min and stored at -80°C until use. Anti-nuclear antibody (ANAs), anti-dsDNA antibody, and anti-Sm antibody concentrations in mouse serum were determined using an ELISA kit (Cusabio Biotech Co., Ltd., Wuhan, China) according to manufacturer instructions. The absorbance at 450 nm was measured using a TriStar LB 941 instrument (Berthold Technologies, Oak Ridge, TN, USA).

Histopathologic examination

Histopathologic analysis to assess renal tubulointerstitial fibrosis was performed on the bilateral kidneys of mice, which were removed after sacrifice, processed by immersion in 10% neutral-buffered formalin and paraffin embedding, and evaluated by Masson staining. The percentage of the entire area dyed blue was calculated and recorded under a microscope as the area of collagen deposition. Renal pathology examinations were performed in a blinded manner by a pathologist.

Flow cytometric analysis

Lymphocytes were collected under sterile conditions from the spleen and peripheral blood, and the lymphocyte suspension was separated by density gradient centrifugation using mouse
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Table 2. Oligonucleotide sequences of exon-specific primer pairs for PCR and sequencing

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward 5'-3' Sequence</th>
<th>Reverse 5'-3' Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>FKN</td>
<td>GGAGATTACTGCCCTGGCTCCTA</td>
<td>GACTCATCGTACTCCTGCTGGCTTG</td>
</tr>
<tr>
<td>IL-17</td>
<td>TGATGCTGTGGTCTGCTGAGAAG</td>
<td>TGGAAACGGTAGCTGAGGAGGA</td>
</tr>
<tr>
<td>IL-6</td>
<td>AGGAGTGGCTAAGGACCAAGAAG</td>
<td>CTGACCACTAGTAGGAACTGCTC</td>
</tr>
<tr>
<td>Foxp3</td>
<td>GAAGAGCCCTGCTTGTACATTGA</td>
<td>TGTGAAGGTCCGATCTGCTGTC</td>
</tr>
<tr>
<td>IL-10</td>
<td>CAAGGCGAATGGAAGCAGGTGAAG</td>
<td>GCTCTGCTACTGCTGAGTCC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>AACTTTGGCATTGTGGAAGG</td>
<td>GGATGCAGGGATGATGTTCA</td>
</tr>
</tbody>
</table>

lymphocyte-separation medium (#P8860; Solarbio) according to manufacturer instructions. The cells were then washed three times with sterile PBS. The lymphocyte suspensions (1 × 10^6 cells/mL) were stained with antibodies specific for mouse CD4, IL-17 (for Th17 cells), and Foxp3 (for Treg cells) or isotype-matched controls according to manufacturer instructions (#560767; BD Biosciences, San Diego, CA, USA). For surface staining, cells were stained with various antibodies at room temperature for 30 min, washed with PBS and then re-suspended and fixed with 1.5% formaldehyde for 2 min under room temperature. For intracellular staining, cells were incubated with PMA (50 ng/mL, Multisciences, China), ionomycin (1 μg/mL, Multisciences, China), and BFA (10 μg/mL, Multisciences, China) for 5 h, then surface stained, fixed, permeabilized, and intracellular stained in accordance with the manufacturer's instruction. Results were further analyzed by FACS Canto II flow cytometer (BD Biosciences).

qRT-PCR

Total RNA from kidney tissue was extracted into RNase-free tubes from Th17-polarized cells and Treg-polarized cells and collected using TRizol reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer instructions. RT was performed with 2 μg of total RNA for cDNA synthesis using the FastKing RT kit (KR116; Tiangen Biotech, Beijing, China).

The primer sequences are shown in Table 2. Expression levels of Fkn, Foxp3, Il17, Il6, Il10, and Gapdh were determined according to SuperReal PreMix Plus (FP205; Tiangen Biotech) instructions and calculated using the 2^-ΔΔCt method, as described previously [19].

Immunohistochemistry (IHC) analysis

Semi-quantitative levels of proteins were analyzed by IHC. Deparaffinized mouse kidney tissue section was blocked in PBS with Tween-20 and containing 0.1% BSA and 10% FBS. Following incubation with primary antibodies against Foxp3 (sc-53876; 1:300; Santa Cruz Biotechnology) and IL-17 (ab79056; 1:300; Abcam) overnight at 4°C, samples were incubated with goat polyclonal secondary antibody (1:200 dilution) for 1 h, developed, and counterstained with hematoxylin. Foxp3 and IL-17 levels in kidney tissues from each group were compared by IHC according to staining intensity and evaluated semi-quantitatively using Image-Pro Plus software (v.5.1; Media Cybernetics Co., Ltd., Rockville, MD, USA) according to a previously described method [20].

Western blot analysis

Mouse kidney tissue (60 mg/mL) from each group and Th17-polarized and Treg-polarized samples from spleen lymphocytes were incubated in radio immunoprecipitation assay buffer (Beyotime Biotechnology, Shanghai, China) and incubated on ice for 30 min, respectively. Primary antibodies against IL-6 (1:500), Foxp3 (1:500), IL-10 (1:500), IL-17 (1:500), FKN (1:3000), and GAPDH (1:1000) were incubated at 4°C overnight, followed by incubation with a goat polyclonal secondary antibody against anti-rabbit or anti-mouse horseradish peroxidase (1:1000) after washing with Tris-buffered saline containing Tween-20 three times. Protein levels were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

Data are presented as the mean ± standard deviation (SD). An unpaired Student’s t test was used to compare the means of two groups. One-way or two-way analysis of variance was used to compare the means of more than two independent groups. All analyses were conducted with SPSS software (v.17.0; SPSS Inc.,
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Figure 1. Serum FKN expression in groups. A. ELISA was performed to assess serum FKN levels in healthy controls, inactive SLE patients, and active SLE patients. B. The correlation between FKN expression and SLEDAI-2K score in patients with inactive and active SLE. C. The correlation between Treg cell numbers and SLEDAI-2K score in patients with inactive and active SLE. D. The correlation between FKN expression and Treg cell numbers in patients with inactive and active SLE. E. The distribution of Treg cells in peripheral blood of groups. **P < 0.01 compared with healthy controls, ##P < 0.01 compared with inactive SLE patients.

Chicago, IL, USA). A P < 0.05 was considered significant.

Results

Clinical characteristics of SLE patients and healthy controls

The demographics and basic clinical characteristics of the patients and controls are presented in Table 1. The serum anti-dsDNA and anti-Sm concentrations in patients with active SLE were significantly higher than those in patients with inactive SLE (P < 0.01), and the decrease in total C4 and C3 concentrations in the SLE group indicated the metabolism of complement components. Additionally, using a SLEDAI-2K score of ≥ 10 to separate active from inactive SLE, a total of thirteen patients were characterized the active disease, while eighteen were characterized with the inactive disease.

Correlation between FKN, Treg differentiation, and SLEDAI score in SLE patients

The abundance of Treg cells in the peripheral blood of healthy controls and SLE patients was analyzed by FC based on established surface markers (Figure 1E), the number of Treg cells significantly decreased in patients with inactive (0.41 ± 0.08) and active SLE (0.09 ± 0.10) compared to those in the healthy controls (0.79 ± 0.08, P < 0.01), and the number of Treg cells was higher in inactive SLE patients than in
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Figure 2. Measurement of marker levels. (A) BUN, (B) serum Cr, (C) 24-h Upro, (D) ANAs, anti-dsDNA antibody, and anti-Sm antibody levels were measured at 13 weeks. NC: C57BL/6 mice; LN: MRL/lpr mice; NC + IgG: NC group treated with an isotype antibody; LN + IgG: LN group treated with an isotype antibody; NC + rFKN: NC group treated with rFKN; LN + rFKN: LN group treated with rFKN; NC + aFKN: NC group treated with aFKN; and LN + aFKN: LN group treated with aFKN. *P < 0.05 vs. the control group; **P < 0.05 vs. the LN group. #P < 0.05, NC + rFKN vs. NC + aFKN; ##P < 0.05, LN + rFKN vs. LN + aFKN.

active SLE patients (P < 0.01). ELISA was used to compare the FKN levels in serum samples from healthy individuals (n=11) with those in samples from SLE inactive patients (n=18) and SLE-active patients (n=13). FKN levels were markedly higher in inactive and active SLE patients than in healthy controls (P < 0.01). Furthermore, the serum levels of FKN in inactive SLE patients were lower than those in active SLE patients (P < 0.01) (Figure 1A). Furthermore, the correlation between the SLEDAI-2K score, serum FKN levels, and the production of Treg cells was demonstrated by simple linear regression. Serum FKN was positively correlated with SLEDAI-2K in both inactive and active SLE patients (r=0.93, P < 0.01) (Figure 1B). In contrast, the number of Treg cells was negatively correlated with the SLEDAI-2K score in both inactive and active SLE patients (r=0.89, P < 0.01) (Figure 1C). Moreover, serum FKN levels in both inactive and active SLE patients were significantly inversely correlated with the Treg cells amount (r=0.95, P < 0.01) (Figure 1D).

Effects of an anti-FKN antibody on renal function

The levels of serum Cr, BUN, urinary protein (Upro), ANAs, anti-dsDNA antibodies, and anti-
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Figure 3. FKN mediates the Treg/Th17 cell ratio in peripheral blood and spleens from MRL/lpr mice. MRL/lpr mice (12-weeks old) were injected i.p. with an isotype antibody, rFKN, or aFKN for 7 days, after which spleens and peripheral blood of LN mice were analyzed by flow cytometry. Cellular distribution for a typical sample...
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Sm antibodies were significantly elevated in the LN group relative to the NC group. Treatment with aFKN decreased these levels (P < 0.05) in the LN + aFKN group as compared with the LN group, although these levels increased in the LN + rFKN group after treatment with rFKN (P < 0.05). No changes in these levels were observed after IgG intervention (P > 0.05) (Figure 2).

Figure 3A and 3B). Additionally, the number of Th17 cells decreased significantly following aFKN treatment (Figure 3B and 3H) but increased significantly following rFKN treatment (Figure 3B and 3F) relative to that observed in the LN group (Figure 3A and 3G). Moreover, the aFKN-treated groups showed a decreased number of Th17 cells (LN + aFKN) and an increased number of Tregs, whereas the number of Tregs decreased in the rFKN-treated group, and the number of Th17 cells increased in the LN + rFKN group. These findings demonstrated that FKN inhibited Treg differentiation and rescued the number of Th17 cells, thereby altering the Treg/Th17 balance and promoted the formation of tubulo-interstitial lesions (TILs) in a murine model of LN. However, no change in the Treg/Th17 ratio was observed after IgG intervention (P > 0.05).

FKN-mediated TILs rely on changes in the Treg/Th17 balance in vivo

We found that FKN significantly regulated protein levels of Foxp3 and IL-17 in kidneys and participated in renal inflammatory injury in MRL/lpr mice. IHC analysis of Foxp3 and IL-17 levels among the different groups indicated decreased glomerular expression of Foxp3 (P < 0.05) and increased IL-17 levels in the LN group as compared with the NC group (P < 0.05) (Figure 4). Furthermore, the LN + rFKN group showed decreased glomerular expression of Foxp3 and increased IL-17 levels as compared with those in the LN group (P < 0.05), whereas IL-17 levels decreased and IL-10 levels increased in the LN + aFKN group (P < 0.05).

Western blot and qRT-PCR results revealed increased mRNA and protein levels of IL-6, IL-17, and FKN (P < 0.05) and decreased levels of Foxp3 and IL-10 in the LN group as compared with those in the NC group (Figure 5). Importantly, mRNA and protein levels of IL-6, IL-17, and FKN in the NC + aFKN group were significantly lower than those in the LN group, whereas Foxp3 and IL-10 levels were higher in the aFKN group. Additionally, mRNA and protein levels of IL-6, IL-17, and FKN in the NC + rFKN group were significantly higher than those in the LN group, whereas levels of Foxp3 and IL-10 in the rFKN group were lower than those in the LN group. However, no significant differences in mRNA and protein levels were observed after IgG intervention (P > 0.05).

A marked increase in aniline blue-positive areas was observed in the renal interstitium in the LN group (P < 0.05) according to Masson staining, and administration of aFKN reduced the size of these areas (P < 0.05) in the LN + aFKN group as compared with the LN group, whereas administration of rFKN increased the size of these areas (P < 0.05) in the LN + rFKN group. However, no change in the size of the blue area was observed after IgG intervention (P > 0.05) (Figure 6).

FKN mediates the inflammatory response of Th17- and Treg-polarized cells by promoting IL-17 and inhibiting Foxp3 expression, respectively, in vitro

Total CD4+ T lymphocytes from splenocytes from the LN group showed markedly higher levels of IL-17 and lower levels of Foxp3 protein and mRNA than those in the NC group (P < 0.05). After re-stimulation, polarized Tregs showed that levels of Foxp3 and IL-10 protein and mRNA were significantly elevated in the
Figure 4. IHC analysis of renal histopathological features of MRL/lpr mice. Original magnification: 400×. NC: C57BL/6 mice; LN: MRL/lpr mice; NC + IgG: NC group treated with an isotype antibody; LN + IgG: LN group treated with an isotype antibody; NC + rFKN: NC group treated with rFKN; LN + rFKN: LN group treated with rFKN; NC + aFKN: NC group treated with aFKN; and LN + aFKN: LN group treated with aFKN. (A) Levels of FKN, IL-17, and Foxp3 according to IHC analysis of renal tissue samples. (B) The column diagram indicates statistical analysis of (A). *P < 0.05 vs. the control group; **P < 0.05 vs. the LN group. #P < 0.05, NC + rFKN vs. NC + aFKN; ##P < 0.05, LN + rFKN vs. LN + aFKN.
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Figure 5. Western blot and qRT-PCR analyses of FKN, IL-17, IL-6, Foxp3, and IL-10 in renal tissue samples. Renal tissue from mice was extracted and subjected to (A) western blot to determine FKN, IL-17, IL-6, Foxp3, IL-10, and GAPDH levels. (B) Total RNA was extracted from renal tissues, and mRNA were quantified by qRT-PCR. GAPDH protein and mRNA were used as internal controls. NC: C57BL/6 mice; LN: MRL/lpr mice; NC + IgG: NC group treated with an isotype antibody; NC + rFKN: NC group treated with rFKN; LN + rFKN: LN group treated with rFKN; NC + aFKN: NC group treated with aFKN; and LN + aFKN: LN group treated with aFKN. *P < 0.05 vs. the control group; **P < 0.05 vs. the LN group. #P < 0.05, NC + rFKN vs. NC + aFKN; ##P < 0.05, LN + rFKN vs. LN + aFKN.

Discussion

Initial results show that flow cytometry analysis and enzyme-linked immunosorbent assay (ELISA) were used to detect the distribution of Treg cells in the peripheral blood and the expression levels of FKN protein in serum separately from 31 patients with LN and 11 healthy controls. Results: Serum level of FKN was significantly increased in SLE patients when compared to controls (P < 0.01). Higher FKN levels were found in active SLE patients when compared with inactive SLE patients (P < 0.01). The distribution of Treg cells in the peripheral blood decreased in SLE patients when compared to controls (P < 0.01), lower FKN levels were found in active SLE patients when compared with inactive SLE patients (P < 0.01). The FKN expression levels were positively correlated with SLEDAI-2K, and negatively correlated with the distribution of Treg cells.

Here, we provided evidence that FKN participates in TIL formation in LN, finding that FKN and IL-17 levels increased along with higher proportions of Th17 cells among total splenocytes in MRL/lpr mice than in C57BL/6 mice and accompanied by decreased levels of Tregs and Foxp3. These results indicated that FKN mediated LN pathogenesis and might be related to Treg/Th17 imbalance. Specifically, aFKN treatment might ameliorate TILs by improving Treg/Th17 balance, inflammation, and fibrosis.
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**Figure 6.** FKN-mediated TILs in MRL/lpr mice. MRL/lpr mice (12-weeks old) were i.p. injected with an isotype antibody, rFKN, or aFKN for 7 days, and (A) pathological changes in renal fibrosis were observed by Masson staining. (B) The column diagram indicates the statistical analysis of (A). *P < 0.05 vs. the control group; **P < 0.05 vs. the LN group. #P < 0.05, NC + rFKN vs. NC + aFKN; ###P < 0.05, LN + rFKN vs. LN + aFKN. No significant difference between the two groups is indicated by &P > 0.05.
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and partially improving Upro excretion and BUN production, suggesting that FKN might also be related to TIL progression in vivo. Additionally, we isolated CD4⁺ T lymphocytes and polarized these cells into two different subsets (Tregs and Th17 cells) via addition of stimulatory factors to cultures of splenocytes from C57BL/6 control and MRL/lpr mice. Our results indicated elevated IL-17, IL-6, and IL-23 levels in the Th17-polarized group and decreased Foxp3, IL-10, and TGF-β levels in the Treg-polarized group following rFKN chemokine stimulation. By con-
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In contrast, aFKN administration had the opposite effects. These findings suggested that FKN modulated lymphocyte inflammation and Treg/Th17 imbalance and ultimately mediated tubulo-interstitial fibrosis in LN.

The MRL/lpr mouse model of LN was established at the Jackson Laboratory in the United States in the 1970s [21]. These mice exhibit lymphoproliferation, develop spontaneous autoimmune-disease symptoms, and present features similar to the pathological changes in human LN, including imbalanced T lymphocyte subsets, autoantibody production, increased lymphocyte infiltration, and splenomegaly [22]. The clinicopathological features that develop spontaneously in MRL/lpr mice are commonly associated with cytokine abnormalities. C57BL/6 mice are the most widely used genetic background for MRL/lpr mice used as models of human disease. Here, we selected C57BL/6 mice as controls.

Typical pathological findings in LN include abnormal lymphocyte aggregation and glomerular destruction. In particular, infiltrating T cells contribute to renal tissue damage in the context of glomerular inflammation, thereby leading to progressive loss of renal function. Studies show that Treg/Th17 imbalance is closely related to autoimmune diseases, such as SLE [23], chronic kidney disease [24], rheumatoid arthritis [25], and atherosclerosis [26]. Inappropriate or unbalanced Treg/Th17 cell ratios underlie several distinct types of autoimmune diseases, including LN, with some of these cells targeting the kidneys by upregulating the production of numerous proinflammatory cytokines, leading to exacerbation of renal injury. CD4⁺CD25⁺Foxp3⁺ Tregs play a key role in controlling and limiting the inflammatory milieu by suppressing cytokine production [8, 27], whereas Th17 cells, as a unique subset of CD4⁺ T cells characterized by IL-17 production, promote tissue inflammation and participate in the development of autoimmune diseases [28, 29]. Regulating immune-cell balance and excessive immune response between T cell subsets helps maintain immune homeostasis and tolerance in vivo. The number of CD4⁺ T cells subsets differs according to immune response and can undergo transformation. For example, naïve CD4⁺ T cells differentiate into Th17 cells after stimulation with IL-6 or IL-21 (together with TGF-β); however, TGF-β drives differentiation into Tregs in the absence of proinflammatory cytokines [30-32]. Additionally, Th17 cells can recruit concentrated granulocytes to promote inflammation at the site of infection by secreting IL-17, IL-22, and IL-23. By contrast, Tregs can inhibit the activity of a variety of immune cells, thereby suppressing the immune response by producing the anti-inflammatory cytokines IL-10 and TGF-β. These findings indicate that Treg/Th17 balance plays a contradictory role during inflammation and immune response.

In this study, we isolated CD4⁺ T lymphocytes from mouse spleen and cultured these cells in the presence of IL-6, TGFβ, IL-23, anti-IFN antibodies, and anti-IL-4 antibodies for Th17 polarization or TGF-β and IL-2 for Treg polarization. We confirmed elevated Foxp3 or IL-17 levels by western blotting, followed by investigation of the role of FKN in the Treg/Th17-polarized subpopulations, respectively.

Similar to the in vivo results, in vitro experiments showed that Foxp3 levels decreased in the Treg-polarized group, whereas increased IL-17 levels were accompanied by increases in levels of the inflammatory factors IL-6 and IL-23 in the Th17-polarized group after rFKN stimulation. We found that Foxp3 expression was restored in the Treg-polarized group, and that IL-17 expression levels were reduced in the Th17-polarized group accompanied by decreased levels of inflammatory factors and elevated IL-10 levels following treatment with aFKN. Our results suggested that FKN had a chemotactic effect on Th17 cells and might decrease the proportion of Tregs by expanding the Th17 subpopulation, inducing Treg/Th17 immune-cell imbalance in the CD4⁺ T lymphocyte population, and thereby affect the immune response.

Increased renal interstitial fibrosis eventually leads to the occurrence of LN. Steinmetz et al. [33] demonstrated that effector Th17 cells mediate glomerulonephritis in MRL/lpr mice. Deficiencies in the C-X-C-motif chemokine receptor 3 lead to improved kidney inflammation and function, with these protective effects mediated by a decrease in Th17 cells in kidney tissue. Yang et al. [34] indicated that MRL/lpr mice showed a reduced degree of inflammation after treatment with an anti-IL-17 antibody, subsequently finding that intervention with IL-17A
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from active SLE patients increased mRNA expression of adhesion molecules and induced adherence in T cells. However, Medina-Contreras et al. [35] found that the enhanced sensitivity of CX3CR1-deficient mice to colitis can be fully reversed by neutralizing IL-17 expression or transferring CX3CR1-expressing macrophages. Therefore, we need to further explore the specific mechanism of action of the FKN receptor CX3CR1 in LN. These findings suggest that different factors have specific CX3CR1 response times and modes, thereby leading to different and even opposing cellular responses.

Notably, this study examined only the role of FKN in regulating TIL formation in LN by regulating Treg/Th17 imbalance. We did not investigate the effect of CX3CR1 silencing on Treg/Th17 imbalance in TILs; therefore, the role of Treg/Th17 cells and the coordination between FKN and CX3CR1 in TILs remain to be investigated.

In summary, these results strongly indicated that SLE bursts are closely related to the expansion of the Th17 cell population and the attenuation of native Treg subpopulations. Our findings suggest that an imbalanced Treg/Th17 population might be closely related to the active expression of the chemokine FKN in LN.

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Disclosure of conflict of interest

None.

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