Lipocalin-2 Exacerbates Lupus Nephritis by Promoting Th1 Cell Differentiation

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ABSTRACT

Background Lipocalin-2 (LCN2) is an indicator of the severity of lupus nephritis (LN) and plays a pivotal role in immune responses, but it is not known if its effect on LN pathogenesis derives from regulating the immune imbalance of T lymphocyte subsets.

Methods The expression of LCN2 in T cells and kidneys was assessed in renal biopsies from patients with LN. We investigated the relationship between LCN2 levels and development of LN and systemic illness by injecting anti-LCN2 antibodies into MRL/lpr mice and analyzing pristane-treated LCN2−/− mice.

Results LCN2 is highly expressed in CD4⁺ T cells and in renal tissues, and is associated with severe renal damage in patients with LN and in mice with experimental lupus. LCN2 promotes IFN-γ overexpression in CD4⁺ T cells through the IL-12/STAT4 pathway in an autocrine or paracrine manner. Both neutralization of LCN2 in MRL/lpr mice and genetic depletion of LCN2 in pristane-induced lupus mice greatly ameliorate nephritis. The frequency and number of splenic and renal Th1 cells decrease in proportion to LN disease activity. Conversely, administration of LCN2 exacerbates the disease with significantly higher renal activity scores and increased numbers of Th1 cells.

Conclusions LCN2 plays a crucial role in Th1 cell differentiation, and may present a potential therapeutic target for LN.

Lupus nephritis (LN), the most relevant manifestation of SLE, is a major contributor to morbidity and mortality.¹ T cells play a pivotal role in SLE pathogenesis, contributing to the initiation and perpetuation of autoimmunity in SLE.² Activated naive CD4⁺ T cells can differentiate into T helper (Th) cells, including Th1, Th2, and Th17, and inducible T regulatory (Treg) cells.³ With abnormal cytokine profiles correlated with loss of immune tolerance and increased antigenic load and defective B cell suppression, Th cells are readily linked to the development of SLE.⁴,⁵ T cells also comprise the majority of kidney-infiltrating cells in lupus with an exhausted phenotype.⁶ Th1 cells in the inflamed kidneys of MRL/lpr mice promote nephritis progression by inducing the deposition of C3,⁷ further supporting the importance of Th cells in LN pathogenesis. Thus, an enhanced understanding of T lymphocyte subset dysfunction in LN and the underlying molecular mechanisms is imperative for novel therapeutic strategies.

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Lipocalin-2 (LCN2), a member of the lipocalin family, has a hydrophobic pocket that binds lipophilic molecules. Initially identified as a 25-kDa secretory glycoprotein in human neutrophil granules, LCN2 is also expressed in other cells such as macrophages and epithelial cells. In addition to initiating innate immune responses, LCN2 modulates cellular immunity and inflammation. In the context of autoimmune disease, LCN2 was instrumental in the pathogenesis of experimental autoimmune encephalitis. Recently, upregulation of LCN2 has been reported to correlate with proteinuria and renal flares in patients with SLE. However, the exact role of increased LCN2 levels in SLE and the underlying molecular mechanism remain poorly understood. LCN2 has been shown to enhance the expression of Th17 cytokines/chemokines in a mouse model of psoriasis. In addition, dendritic cell–secreted LCN2 participates in T cell priming and promotes a Th1 microenvironment, further suggesting that LCN2 plays a pivotal role in immune responses. Nevertheless, whether LCN2 exerts its effect on LN pathogenesis by influencing T lymphocytes has yet to be elucidated.

In this study, LCN2 is found to be highly expressed in naive CD4+ T cells and kidneys in patients with LN and LN mice. We report here that administration of LCN2 aggravates kidney damage and promotes expansion of Th1 cells. However, removing LCN2 prevents the development of LN with decreased Th1 cells. LCN2+/− T cells show decreased IL-12–induced signal transducer and activator of transcription 4 (STAT4) phosphorylation that is required for Th1 cell differentiation in vitro. Together, our findings demonstrate that LCN2 plays a key role in regulating Th1 differentiation. The study highlights that intervention of LCN2 could be an effective therapy of LN by inhibiting Th1 response.

METHODS

Patients
A total of 18 patients with SLE without LN, 46 patients with SLE and LN (Supplemental Table 1), and 34 age- and sex-matched healthy donors recruited from Nanjing Drum Tower were enrolled in this study. The diagnosis of SLE was based on the American College of Rheumatology revised classification criteria for SLE. Urine samples were centrifuged at 2500 × g (4°C) for 30 minutes to remove cellular debris before storing. Urine and serum samples were frozen within 2 hours after collection and stored at −80°C until further analysis. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll density-gradient centrifugation. Blood tests, including assays to determine 24-hour proteinuria and serum creatinine, were performed using standard methods. Naïve human CD4+CD45RA+ T cells were purified by magnetic cell sorting (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s instructions. Fluorochrome-labeled CD14+ monocytes, CD11c+ dendritic cells (DCs), CD19+ B cells, CD3−CD56+ natural killer (NK) cells, CD3+CD4+CD45RA+CD62L+ naïve CD4 T cells, and CD3+CD8+CD45RA+CD62L+ naïve CD8+ T cells were sorted using a FACSAria cell sorter (BD Biosciences, Franklin Lakes, NJ). Purity was around 96%. Renal biopsy samples from 25 patients with LN that were allocated to SLE classes 2–5, according to the classification of the International Society of Neurology–Renal Pathology Society, and six patients with diabetic nephropathy (DN) were enrolled in the study. Seven normal kidney tissue samples adjacent to renal carcinoma served as normal controls. An overview of the composition and clinical data for the patients with LN is provided in Supplemental Table 2. All diagnoses were made by expert renal pathologists.

Microarray Analysis
Gene expression profiles were analyzed with the human 1.0ST GeneChip (Affymetrix, Santa Clara, CA), strictly following the manufacturer’s protocol. Microarray experiments were performed at Genmixin Informatic Ltd. (Shanghai, China) with the microarray service certified by Affymetrix. The gene expression microarray data have been submitted to the Gene Expression Omnibus database with accession number GSE144390.

Animals
C57BL/6 (B6) female mice were purchased from Nanjing Medical University Animal Core (Nanjing, China). B6.LCN2+/− mice were purchased from Jackson Laboratories (Bar Harbor, Maine). Female MRL/lpr mice were obtained from Shanghai Lingchang Biotechnology Corporation (Shanghai, China).

Treatment of Mice
B6 and B6.LCN2+/− female mice were treated with a single intraperitoneal injection of 0.5 ml pristane (Sigma-Aldrich, St. Louis, MO) per mouse at the age of 8–10 weeks. Control groups of mice of the above strains were injected with the same volume of PBS. The 16-week-old MRL/lpr mice received weekly i.p. injections of 100 μg of a blocking monoclonal LCN2 antibody (clone 228418; R&D Systems, Minneapolis, MN) or purified rat IgG2a isotype control antibody (R&D Systems) for 4 weeks. The 16-week-old MRL/lpr
mice received weekly i.p. injections of 10 μg recombinant LCN2 protein (R&D Systems) or PBS for 4 weeks.

Evaluation of Renal Injury
Spot urine was collected at the same time every week using the “bladder-message” method.\textsuperscript{18,19} Urinary albumin was determined using an Albumin Assay Kit (Jiancheng, Nanjing, China) and standardized by urine creatinine concentration. The concentrations of creatinine in serum and urine were measured using the Creatinine Assay Kit (Jiancheng) according to the manufacturer’s instructions.

For renal histopathology assessment, kidneys were fixed in 10% neutral buffered formalin, embedded in paraffin, and cut into 3-μm sections. Sections were stained with hematoxylin and eosin and Periodic acid–Schiff. We evaluated kidney pathology as previously described.\textsuperscript{20,21} Briefly, glomerular pathology was assessed by examining 20 glomerular cross-sections (gcs) per kidney and scoring each glomerulus on a semiquantitative scale as follows: 0, normal (35–40 cells/gcs); 1, mild (glomeruli with few lesions showing slight proliferative changes, mild hypercellularity [41–50 cells/gcs], and/or minor exudation); 2, moderate (glomeruli with moderate hypercellularity [51–60 cells/gcs], including segmental and/or diffuse proliferative changes, hyalnosis, and moderate exudates); and 3, severe (glomeruli with segmental or global sclerosis and/or severe hypercellularity [>60 cells/gcs], necrosis, crescent formation, and heavy exudation). Interstitial/tubular pathology was assessed semiquantitatively on a scale of 0–3 in ten randomly selected high-power fields (×400). We determined the largest and average number of infiltrates and damaged tubules and adjusted the grading system accordingly: 0, normal; 1, mild; 2, moderate; and 3, maximum. Perivascular cell accumulation was determined semiquantitatively by scoring the number of cell layers surrounding the majority of vessel walls on a 0–3 scale (0, none; 1, less than five cell layers; 2, five to ten cell layers; and 3, more than ten cell layers).

For the immunofluorescence evaluation of IgG and C3 deposits in the kidneys, kidney tissues were embedded with optimal cutting temperature compound. Frozen sections were stained with FITC–anti-mouse IgG or FITC–anti-mouse C3 (1:50, Santa Cruz Biotechnology, Santa Cruz, CA) as previously described.\textsuperscript{22,23} Glomerular IgG and C3 staining was graded according to intensity on a scale of 0–4 as follows: 0, absent; 1, faint; 2, moderate; 3, intense; and 4, very intense.

ELISA and Biochemical Parameters
Serum anti–double stranded DNA antibody (anti-dsDNA Ab) levels and supernatant IFN-γ levels were measured using the mouse anti-dsDNA ELISA Kit (Shibayagi, Gunma, Japan) and mouse IFN-γ ELISA kit (4A Biotech Co., Ltd, Beijing, China), respectively, according to the manufacturer’s instructions.

Real-Time PCR
RNA was extracted from tissues or cells using Trizol (Vazyme Biotech, Nanjing, China). For cDNA synthesis, reverse transcription was performed from 2 μg of total RNA using the HiScript II Q RT SuperMix (Vazyme Biotech). Quantitative real-time PCR assays were performed using the SYBR Green Master Mix (High ROX Premixed) and the StepOnePlus Real-Time PCR Systems (Applied Biosystems, Foster City, CA). The relative expression of each gene was determined and normalized to the expression of housekeeping gene glyceraldehyde 3-phosphate dehydrogenase using the $^{\Delta\Delta}\text{Ct}$ method. Gene-specific primers (Genscript Biotech, Nanjing, China) are listed in Supplemental Table 3.

Immunohistochemistry
Sections (3 μm) of formalin-fixed, paraffin-embedded tissue specimens were mounted on glass slides, dewaxed, rehydrated, immersed in citrate buffer (0.01 M, pH 6.0), and then a heat-mediated antigen retrieval procedure was performed. The sections were then incubated with anti-LCN2 antibody (1:8000, ab206427; Abcam, Cambridge, United Kingdom) or anti-CD68 antibody (1:100, ab31630) or anti-Ly6G (anti–Gr-1) antibody (1:800, ab238132) overnight at 4°C. The secondary antibody was incubated for 30 minutes at 37°C. Negative controls were included each time. The immunostaining was developed using diaminobenzidine tetrahydrochloride, followed by counterstain with Mayer hematoxylin. We counted the number of cells showing positive expression for LCN2, CD68, and Gr-1 in high-power fields.

Immunofluorescence
Paraffin sections were deparaffinized, rehydrated, and boiled in citrate buffer (pH 6.0) to retrieve antigens. Paraffin sections and frozen sections were permeabilized, blocked, and incubated with the indicated primary antibodies at 4°C overnight. The primary antibodies used were as follows: goat anti–LCN2 antibody (catalog number AF1757; R&D Systems), rabbit anti-CD3 antibody (1:50, ab16669; Abcam), rabbit anti-CD68 antibody (1:100, ab125212), mouse anti-CD15 antibody (1:100, clone 4E10; Novus Biologicals, Littleton, CO), mouse anti–E–Cadherin antibody (1:100, clone M168; Abcam), and Alexa Fluor 594 anti-mouse Gr-1 antibody (1:50, clone RB6–8C5; BioLegend, San Diego, CA). Subsequently, the sections were incubated with secondary antibodies for 1 hour at room temperature. The secondary antibodies used were as follows: Alexa Fluor 488–conjugated donkey anti-goat IgG, Alexa Fluor 594–conjugated goat anti-rabbit IgG, and Alexa Fluor 594–conjugated goat anti-mouse IgG (Thermo Fisher Scientific, Fremont, CA). The secondary antibodies were prepared in 1% BSA dissolved in PBS buffer. Sections were kept in a dark environment. The colocalization of LCN2 with other biomarkers was demonstrated by confocal laser scanning microscopy using a Olympus FV3000 Microscope. Mean fluorescence intensity was quantified using ImageJ software (National Institutes of Health, Bethesda, MD).

Isolation of Leukocytes from Various Tissues
Peripheral blood was drawn into EDTA-coated tubes. PBMCs were isolated using Ficoll density-gradient centrifugation.
Figure 1. LCN2 expression is elevated in CD4\(^+\) T cells and the kidneys of patients with LN. (A) Screening of the top 20 upregulated genes in PBMCs from patients with SLE (SLE; \(n=3\)) and healthy controls (C; \(n=3\)). (B) Real-time PCR validation of gene expressions in PBMCs from patients with SLE (\(n=6\)) and healthy control (HCs; \(n=6\)). (C) Expression of LCN2 in naive CD4\(^+\) T cells derived from patients with SLE and LN (\(n=14\)), patients without LN (non LN, \(n=16\)), and HCs (\(n=20\)). (D) Immunohistochemical staining of LCN2 in the kidneys from patients with class 2 LN (\(n=5\)), class 3 LN (\(n=5\)), class 4 LN (\(n=8\)), class 4+5 (\(n=3\)), and class 5 (\(n=4\)), respectively. Quantification of LCN2-positive area is shown in the right panel. (E) The data plots show the correlation analysis of LCN2-positive area in kidneys with the active index, chronic index, and interstitial inflammation in patients with LN (\(n=25\)). \(P\) values are determined by Mann–Whitney \(U\) test in (B), one-way ANOVA with Tukey multiple comparisons test in (C), and one-way ANOVA with Dunnett multiple comparisons test in (D). Both correlation coefficient \(r\) and \(P\) values are calculated by the Spearman \(r\) test in (E). Data are shown as mean±SEM. *\(P<0.05\), **\(P<0.01\), ***\(P<0.001\), ****\(P<0.0001\). Original magnification, \(\times 100\). Ctrl, control.
Spleens and mesenteric lymph nodes were removed with scissors. Single-cell suspensions were prepared by teasing the organs in a glass homogenizer with a loosely fitting pestle.

Erythrocytes in spleen cell suspensions were lysed with red blood cell lysis buffer, and cells were washed and passed over 40-μm meshes. Cells were then washed again, counted, and

**Figure 2.** LCN2 expression is increased in T cells, macrophages, neutrophils, and renal TECs in LN kidneys. Representative photos of immunofluorescence staining of LCN2 and different cell type markers in renal biopsy sections of controls (Ctrl), patients with LN, and those with DN. (A) LCN2+ cells (green) and CD3 (red) are stained. (B) LCN2+ cells (green) and CD68 (red) are stained. (C) LCN2+ cells (green) and CD15 (red) are stained. (D) LCN2+ cells (green) and E-cadherin (red) are stained. Colocalization is visualized by the yellow merge of red and green signals. Nuclei are stained with 4',6-diamidino-2-phenylindole (blue). (E) Quantification of LCN2 immunofluorescence intensity in the experiments of (A–D) (n=6). One-way ANOVA with Dunnett multiple comparisons test is used. Data are shown as mean±SEM. *P<0.05, **P<0.01. Original magnification, ×100. Mφ, macrophages; Neu, neutrophils; T, T cells; TEC, tubular epithelial cells.
resuspended in PBS for culture or FACS analysis. Kidneys were digested for 45 minutes at 37°C by adding 1 ng/ml collagenase IV (Sigma-Aldrich) and 0.01 mg/ml DNase I (Sigma-Aldrich) to RPMI 1640 (Gibco Life Technologies) medium containing 10% FBS. Tissues were then homogenized, passed over 40-μm nylon meshes, and centrifuged at 300 × g at 4°C for 10 minutes. After lysis of erythrocytes with red blood cell lysis buffer, single-cell suspensions were separated using Percoll density-gradient centrifugation. Cells were washed, counted, and re-suspended in PBS for staining and FACS analysis.

Flow Cytometry
Cells were incubated in PBS containing 0.5% BSA. The following antibodies were used in this study: FITC anti-mouse CD4, PE anti-mouse IL-4, PerCP anti-mouse IFN-γ, APC anti-mouse IL-17A, PE anti-mouse Foxp3, APC anti-mouse CD25, PE anti-mouse PD-1, APC anti-mouse CXCR5, PerCP anti-mouse CD45, APC anti-mouse IFN-γ, FITC anti-mouse B220, APC anti-mouse CD3, PE anti-mouse NK1.1, PerCP anti-mouse CD4, FITC anti-mouse CD8, PE anti-mouse IL-17A, and FITC anti-mouse IL-4. All antibodies were purchased from eBioscience (San Diego, CA). For cytokine detection, cells were stimulated with 50 ng/ml phorbol 12-myristate 13-acetate plus 1 ng/ml ionomycin and 5 μg/ml brefeldin A at 37°C for 4 hours. For intracellular cytokine staining, cells were fixed and permeabilized with a fixation/permeabilization kit according to the manufacturer's protocol (eBioscience). Data were acquired by a FACS Calibur flow cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star, Ashland, OR).

In Vitro Th1 Cell Differentiation
Naive CD4+ T cells from spleens were purified using the EasySep kit (STEMCELL Technologies, Vancouver, BC, Canada). Purified cells were cultured with plate-bound anti-CD3 (2.5 μg/ml; eBioscience) and anti-CD28 (5 μg/ml; eBioscience) for 5 days under Th1-polarizing conditions, and with IL-12 (10 ng/ml; PeproTech, Rocky Hill, NJ), IL-2 (10 ng/ml), and anti–IL-4 (10 μg/ml; eBioscience) for Th1 cell polarization.

Western Blot Analysis
Purified CD4+ T cells lysed in radioimmunoprecipitation assay buffer with a Halt Protease Inhibitor mixture (Cell Signaling Technology, Beverly, MA) and PMSF (Beyotime Biotech, Jiangsu, China). Anti-human LCN2 (0.2 μg/ml, catalog number AF1757), anti-mouse LCN2 (0.25 μg/ml, catalog number AF1857; R&D Systems), anti–STAT4 (1:1000; Cell Signaling Technology), anti–phosphorylated-STAT4 (1:100; Santa Cruz Biotechnology), anti–LCN2 receptor (anti–24p3R; 1:1000; ImmunoWay, Newark, DE), and anti–glyceraldehyde 3-phosphate dehydrogenase (1:1000) antibodies were used to probe the blots according to standard procedures. The proteins were visualized with enhanced chemiluminescence (Millipore).

Statistical Analyses
All values are expressed as the mean ± SEM. We assessed data for normal distribution and similar variance between groups. Statistical analyses were performed using two-tailed unpaired t tests for comparisons between two groups. One-way ANOVA was used for multiple comparisons. When the data were not normally distributed or displayed unequal variances between two groups, we used the Mann–Whitney U test for statistical analysis. Correlation analysis was performed using the Spearman r test. For survival analysis, the log-rank test was used to compare survival curves. We used SPSS software version 23.0 (IBM SPSS; Chicago, IL) and GraphPad Prism version 7.0 (GraphPad Software, San Diego, CA) for statistical analysis. A P value <0.05 was considered to be statistically significant.

Study Approval
All human subjects were approved by the ethics committee of the Affiliated Drum Tower Hospital of Nanjing University Medical School (2016-027-01). Written informed consent was obtained from all subjects. All experimental protocols were approved by the Ethics Committee for Animal Research in the Affiliated Drum Tower Hospital of Nanjing University Medical School, and all experiments were conducted in compliance with guidelines for the care and use of laboratory animals and approved by the Institutional Animal Care and Use Committee of the Affiliated Drum Tower Hospital of Nanjing University Medical School (20161205).

RESULTS
LCN2 Expression Is Increased in SLE CD4+ T Cells
To determine the differentially expressed genes in SLE PBMCs, RNAs purified from fresh PBMCs of patients with SLE and healthy control subjects were hybridized to a GeneChip Human Gene 1.0 ST Array. According to the microarray data, the top 20 upregulated genes in SLE PBMCs were listed (Figure 1A). To confirm the reliability of the microarray data, five upregulated genes (OLFM4, DEFA4, LCN2, CRISP3, and RNASE3; fold change, >10) were chosen, and the expression of these genes was confirmed in another set of PBMCs. Of these genes, increased OLFM4, DEFA4, LCN2, and CRISP3 expression was confirmed in SLE PBMCs, with LCN2 showing the highest increase (Figure 1B). We performed a comparative analysis of the protein levels (Supplemental Figure 1) of LCN2 in human peripheral blood monocytes, DCs, T cells, B cells, and NK cells. The LCN2 expression level in naive CD4+ T cells was comparable to that of DCs and monocytes. We further examined LCN2 expression in naive CD4+ T cells from patients with SLE. The mRNA level of LCN2 in patients with LN was significantly increased compared with that in healthy control subjects and patients without LN (Figure 1C). Moreover, the LCN2 expression levels in naive CD4+ T cells were positively
correlated with SLE Disease Activity Index scores, urine protein, and serum creatinine levels in patients with SLE (Supplemental Figure 2, A–C). MRL/lpr mice developed typical LN-like symptoms, with increasing deposition of IgG and albuminuria levels (Supplemental Figure 3, A and B). Consistent with the results for human samples, 15- or 20-week-old mice expressed significantly higher levels of LCN2 in PBMCs and splenic CD4+ T cells than 10-week-old mice (Supplemental Figure 3, C and D), suggesting that LCN2 levels increased with the progression of LN. These data implied a potential role for LCN2 in CD4+ T cell–mediated immune dysfunction in LN.

Elevated Renal LCN2 Levels Correlate with Nephritis Severity in Patients with LN and in MRL/lpr Mice

To determine whether LCN2 expression is also upregulated in the kidneys from patients with LN, we studied kidney biopsy samples from patients with different LN classes (Supplemental Table 2). The percentage of LCN2-positive area was significantly higher in class 4 and 5 compared with class 1–3 (Supplemental Figure 3, E). These data suggested that LCN2 expression was positively correlated with the severity of LN.

Figure 3. LCN2 accelerates the development of LN. (A) Albuminuria is expressed as micrograms of albumin per milligram of urinary creatinine during the course of treatments in LCN2+ (n=6) or PBS– (n=7) treated mice. (B) Spleen/body weight ratio of both groups. (C) Immunofluorescence staining for mouse IgG and C3. Original magnification, ×400. (D) Representative photographs of kidney sections stained with hematoxylin and eosin (HE) and Periodic acid–Schiff (PAS). Original magnification, ×100. Quantification of glomerular, tubulointerstitial, and perivascular pathology. (E) The mRNA expression of IFN-γ, IL-6, TNFα, IL-1β, and MCP-1 in the kidneys. (F) Representative photographs of CD68 and Gr–1–stained kidney sections from LCN2+ or PBS–treated mice. Original magnification, ×400. Quantification of positive cells per high-power field (HPF) is shown in the right panels. (G) Representative flow cytometric analysis of Th1 cells in spleens and lymph nodes from mice treated with LCN2 or PBS. The percentage and number of Th1 cells are shown in the right two panels, respectively. Data are representative of two independent experiments. P values are determined by two-tailed unpaired t test in (A–D, F, and G) and by Mann–Whitney U test in (E). Data are shown as mean±SEM. *P<0.05, **P<0.01, ***P<0.001.
Figure 4. LCN2 deficiency prevents LN development by suppressing Th1 cells. (A) Percentage survival, showing animals euthanized due to pristane treatment (n=6 for WT or LCN2−/− mice, n=20 for WT+pristane or LCN2−/−+pristane mice). (B) The spleen/body weight ratio from PBS-treated WT (n=6) and LCN2−/− mice (n=6) and pristane-treated WT (n=14) and LCN2−/− mice (n=20). (C) Albuminuria and (D) serum creatinine levels from each treatment group. (E) Representative photographs of kidney sections stained with hematoxylin and eosin (HE) and Periodic acid–Schiff (PAS). Original magnification, ×100. Quantification of glomerular, tubulointerstitial, and perivascular pathology. (F) Photos and graphs of IgG and C3 deposition in glomeruli. Original magnification, ×400. (G and H) Representative flow cytometric analysis of Th1 cells in (G) splenic and (H) renal (n=4 for each group) CD4+ T cells. The percentage and number of Th1 cells are shown in the right two panels, respectively. Data are representative of three independent experiments. P values are determined by one-way ANOVA with Dunnett multiple comparisons test in (B–D, G, and H) and by two-tailed unpaired t test in (E and F). Data are shown as mean±SEM. *P<0.05, **P<0.01, ***P<0.001.

Consistently, both the mRNA and protein levels of LCN2 in the kidney were increased with the progression of LN in MRL/lpr mice (Supplemental Figure 3, E and F). These data suggest that increased LCN2 expression may contribute to nephritis development in SLE pathogenesis. To find out which cell types express LCN2 in the kidney of patients with LN, we performed dual-color immunofluorescence analyses of LCN2 and a panel of phenotypic cell markers. LCN2 was found colocalized with CD3+ T cells (Figure 2A), CD68+ macrophages (Figure 2B), CD15+ neutrophils (Figure 2C), and E-cadherin+ tubular epithelial cells (TECs) (Figure 2D). The average fluorescence intensity of LCN2 in each cell type was also compared in the kidney of patients with LN versus controls and patients with DN (diabetic nephropathy) (Figure 2E). Compared with
Figure 5. Administration of anti-LCN2 antibodies ameliorates the pathologic phenotype of lupus mice. (A) Percentage survival of mice treated with anti-LCN2 (n=8) or control antibodies (n=10). (B) Albuminuria during the course of treatments in mice treated with anti-LCN2 or control antibodies. (C) Spleen/body weight ratio in mice treated with anti-LCN2 (n=8) or control antibodies (n=8). (D) Representative photographs of kidney sections stained with hematoxylin and eosin (HE) and Periodic acid–Schiff (PAS). Original
controls, we found much higher intensity of LCN2 in macrophages and TECs in both LN and DN, whereas the higher intensity of LCN2 in T cells and neutrophils was only found in LN, implying a different cellular source of increased LCN2 in LN and other kidney diseases. LCN2 was also found to be expressed in T cells, macrophages, neutrophils, and TECs in MRL/lpr mice (Supplemental Figure 3G).

**LCN2 Accelerates the Development of LN**

To investigate the functional relevance of LCN2 upregulation in the development of LN, the 16-week-old MRL/lpr received weekly i.p. injections of 10 μg recombinant LCN2 protein or PBS for 4 weeks. The survival rate showed no significant difference between the two groups (Supplemental Figure 4A). Albuminuria increased significantly at 20 and 21 weeks (Figure 3A). The splenic index (spleen/body weight ratio) was increased (Figure 3B). Anti-dsDNA Ab and serum creatinine showed no significant change after treatment of MRL/lpr mice with recombinant LCN2 (Supplemental Figure 4, B and C). Histologic analysis of the kidneys was performed in a blinded manner to evaluate the severity of LN. The application of LCN2 significantly increased glomerular C3 depositions and the histiocyte score (Figure 3, C and D). Kidney histopathology showed severe glomerular, interstitial, and vascular lesions in LN-LCN2-treated mice, which were characterized by proliferative GN as well as tubulointerstitial inflammation. To study the effect of LCN2 on proinflammatory cytokines, we analyzed mRNA expression of IFN-γ, IL-6, TNFα, IL-1β, and MCP-1 in the kidney. As Figure 3E showed, LCN2 significantly increased mRNA expression of these cytokines. To assess whether LCN2 might affect the renal infiltration of leukocyte subsets, we performed immunohistochemical staining of kidney sections. Staining for CD68+ macrophages and Gr-1+ neutrophils showed a significant increase in the kidney of LN-LCN2-treated mice (Figure 3F). We then obtained splenic and mesenteric lymph node cells to assess the in vivo effects of LCN2 on the imbalance of Th cell subsets in MRL/lpr mice. As shown in representative FACs plots, injection of LCN2 increased the frequency and number of Th1 cells in the spleen and lymph node (Figure 3G). The percentages of Treg, Th2, and Th17 cells in the spleen and lymph node (Supplemental Figure 4, D and E) showed no obvious differences. These results indicate that LCN2 upregulation promotes and aggravates the development of LN.

**LCN2 Deficiency Prevents LN Development by Suppressing Th1 Cells**

To further clarify the role of LCN2 in LN development, LCN2−/− mice were used. The LCN2−/− mice were born normally and showed no obvious abnormalities in body size and weight or in lymphocyte populations compared with those of age-matched wild-type (WT) mice (Supplemental Figure 5). We then induced a lupus model in WT and LCN2−/− mice by i.p. injection of pristane. As shown in Figure 4A, five out of 20 WT mice died within the first month and one mouse died at 4.5 months after injection of pristane. Alveolar hemorrhage was responsible for the early mortality induced by pristane. Compared with pristane-treated WT mice, pristane-treated LCN2−/− mice exhibited an improved survival rate. In addition, splenomegaly was not obvious in pristane-treated LCN2−/− mice (Figure 4B). Albuminuria and serum creatinine (Figure 4, C and D) both decreased in pristane-treated LCN2−/− mice. The histologic parameters were analyzed to compare the clinical outcome in WT and LCN2−/− mice upon pristane induction. Quantification of the renal tissue damage, in terms of glomerular and tubulointerstitial injury, revealed a reduction in LCN2−/− mice (Figure 4E). In line with these results, we also found a significant reduction in glomerular IgG and C3 deposits (Figure 4F). Immunohistochemical staining of CD68 and Gr-1 in the kidney revealed a reduction of macrophage and neutrophil infiltration in LCN2-deficient, lupus-prone mice (Supplemental Figure 6A). Taken together, these results indicate that LCN2 deficiency prevents LN development. Furthermore, we also investigated T cell responses in the spleen. Compared with PBS treatment, pristane treatment increased the proportions of Th1, Th2, Th17, and Treg cells. However, only Th1 cells showed a decrease in pristane-treated LCN2−/− mice compared with those in pristane-treated WT mice. Both the frequency and number of Th1 cells were significantly reduced (Figure 4G). There were no significant differences in Th2, Th17, Treg, or T follicular helper cells between pristane-treated LCN2−/− and WT mice (Supplemental Figure 6B). We also obtained a single-cell suspension of kidneys to detect Th1 cells. It was expected that more Th1 cells would have infiltrated the kidney in pristane-treated WT mice. Notably, however, pristane-treated LCN2−/− mice had a reduced frequency and number of Th1 cells in the kidney compared with those of pristane-treated WT mice (Figure 4H). Together, these findings indicate that the aberrant differentiation of Th1 cells induced by LCN2 plays an

Magnification, ×100. Quantification of glomerular, tubulointerstitial, and perivascular pathology. (E) Photos and graphs of IgG and C3 deposition in glomeruli. Original magnification, ×400. (F) Representative photographs of CD68- and Gr-1−-stained kidney sections. Original magnification, ×400. Quantification of positive cells per high-power field (HPF) is shown in the right two panels. (G) The mRNA expression of IFN-γ, IL-6, TNFα, IL-1β, and MCP-1 in the kidneys. (H) Representative flow cytometric analysis of Th1 cells in the spleens and lymph nodes from mice treated with anti-LCN2 or control antibodies. The percentage and number of Th1 cells are shown in the right two panels, respectively. Data are representative of two independent experiments. P values are determined by two-tailed unpaired t test in (B–E), the top scatter plot diagram in (F), IFN-γ, IL-6, and MCP-1 in (G), and the percentage of Th1 cells in (H); and by Mann–Whitney U test in the lower scatter diagram in (F), TNFα and IL-1β in (G), and the number of Th1 cells in (H). Data are shown as mean±SEM. *P<0.05, **P<0.01, ***P<0.001.
important role in the pathogenesis of LN, and LCN2 deletion may be responsible for the notable improvement of pristane-induced LN.

Administration of Anti-LCN2 Antibodies Ameliorates the Pathologic Phenotype of Lupus Mice

Our findings revealed the important role of LCN2 in LN pathogenesis. Therefore, we sought to evaluate the efficacy and potential application of anti-LCN2 antibody as treatment for LN. Compared with rat IgG-treated animals, anti-LCN2 antibody–treated MRL/lpr mice exhibited a mild improvement in survival rate (Figure 5A). At the end of the experiment (20 and 21 weeks), the albuminuria and splenic index (Figure 5, B and C) were significantly decreased in the anti-LCN2 antibody group. There was no significant difference for anti-dsDNA Ab and serum creatinine (Supplemental Figure 7, A and B). Histologic analysis showed less cellular proliferation and mesangial matrix deposition in glomeruli and improved interstitial lesions (Figure 5D). Neutralization of LCN2 also markedly improved the glomerular deposition of C3 (Figure 5E). Quantification of CD68+ cells and Gr-1+ cells revealed a significant decrease in renal macrophages and neutrophils in the anti-LCN2 antibody group (Figure 5F). mRNA expression of IFN-γ, IL-6, TNFα, and IL-1β also decreased greatly (Figure 5G). Notably, anti-LCN2 antibodies markedly reduced the percentage and number of Th1 cells in both spleen and lymph node cells of MRL/lpr mice (Figure 5H). In contrast to the remarkable depressive effects of anti-LCN2 antibodies on Th1 cell differentiation, the frequencies of Treg, Th2, and Th17 cells were not significantly affected (Supplemental Figure 7, C and D). Taken together, these data indicate that neutralization of LCN2 expression in vivo inhibits the Th1 cell response in LN and could effectively ameliorate disease severity.
LCN2 Promotes Th1 Cell Differentiation via the IL-12-STAT4 Axis

To investigate the mechanism underlying LCN2 regulation of Th1 cell differentiation, WT and LCN2$^{-/-}$ naive CD4$^+$ T cells, sorted by magnetic cell sorting, were differentiated into Th1 cells under specific skewing conditions. The ablation of LCN2 in CD4$^+$ T cells was confirmed (Supplemental Figure 8). Macrophage cell line (RAW264.7) stimulated with or without LPS (10 µg/ml) served as positive and negative controls, respectively (Supplemental Figure 8B). Under the condition of Th1 cell differentiation, the frequency of Th1 cells generated from LCN2$^{-/-}$ naive CD4$^+$ T cells was significantly lower than that from WT T cells. LCN2$^{-/-}$ naive CD4$^+$ T cells were treated with recombinant LCN2 protein (0.1–10 µg/ml), and the frequency of Th1 was assessed. Th1 cell generated from LCN2$^{-/-}$ naive CD4$^+$ T cells significantly increased after treatment with 1 and 10 µg/ml LCN2 protein (Figure 6A). IFN-γ levels in the culture supernatants were significantly lower in LCN2$^{-/-}$ Th1 cells under Th1 cell polarization than in WT T cells and LCN2$^{-/-}$ T cells treated with recombinant LCN2 protein (1 µg/ml) (Figure 6B). We also detected the expression of LCN2 receptor (24p3R) in naive CD4$^+$ T cells (Figure 6C). The molecular network of Th1 cell differentiation involves multiple essential transcription factors, including members of the STAT family. In particular, STAT4 is essential for Th1 cell differentiation derived from naive T cells in response to IL-12. We therefore addressed the involvement of LCN2 in the activation of STAT4 in this network. IL-12–induced STAT4 phosphorylation was diminished in LCN2$^{-/-}$ T cells, which was partially reversed by the addition of LCN2 (Figure 6D). In addition, LCN2$^{-/-}$ Th1 cells expressed lower levels of IL-12 receptor β2 (IL12Rβ2), T-bet, and IFN-γ (Figure 6E), indicating LCN2 is a positive regulator of the expression of genes relating to Th1. We also examined the effects of a neutralizing antibody against LCN2 on Th1 cell differentiation from naive T cells of MRL/lpr mice. As Figure 6F showed, in vitro differentiated Th1 cells from MRL/lpr mice decreased significantly after neutralization by anti-LCN2 antibody (10 µg/ml). These data suggest that LCN2 promotes Th1 cell differentiation through the IL-12/STAT4 pathway.

**DISCUSSION**

In this study, we show that LCN2 is upregulated in SLE and contributes to the immune imbalance of LN by promoting the differentiation of Th1 cells in vitro and in vivo. Additionally, removing LCN2 prevents the development of nephritis in lupus mice. Therefore, LCN2 is involved in LN development and acts as a driver of extraordinary expansion of Th1 cells.

LN can cause permanent kidney damage. In response to renal injury, LCN2 is upregulated in resident kidney cells, as demonstrated in patients with acute nephrotic damage or proliferative GN. Consistently, we found that patients with all classes of LN showed more intense expression of LCN2 in the kidneys than controls. The numbers of LCN2$^+$ cells were also positively correlated with the active index, a predictor of rapid progression to renal failure. These data suggest that LCN2 overexpression contributes to kidney injury in LN. Pathogenic anti-dsDNA Ab can modify gene expression in lupus mesangial cells, including upregulated expression of LCN2. SLE is characterized by pathogenic autoantibody production. On the MRL background, the lpr mutation leads to the development of various autoantibodies against dsDNA, Smith, and circulating immune complexes. Thus, autoantibodies may be important contributors to the overexpression of LCN2 in SLE.
Schiffer et al. reported that the increased LCN2 in the kidneys of NZB/W mice was caused by intrinsic renal cells. Consistently, we found LCN2 was highly expressed in TECs of patients with LN. Interestingly, the infiltrated leukocytes—including T cells, macrophages, and neutrophils—were also responsible for the increase of LCN2 in the kidney. Higher levels of LCN2 in renal T cells and neutrophils were specifically found in patients with LN, indicating a distinct cellular source of LCN2 in different kidney diseases. In addition to the kidney, we identified PBMCs as an extrarenal source of increased LCN2 in SLE. Moreover, the expression of LCN2 from naive CD4+ T cells of patients with LN was markedly increased. To our knowledge, there is little evidence that T cells can express LCN2, although its source from innate immune cells has been extensively studied. Itial role in the development of the immune system. Floderer et al. demonstrated a direct effect of LCN2 on CD4+ T cells was confirmed by using specific antibodies, further reinforcing the validity of our findings.

T cell signaling abnormalities contribute to aberrant immune cell function and SLE. In this study, LCN2 was found to promote Th1 cell differentiation in autocrine/paracrine manners in vitro. The findings were also confirmed in MRL/lpr mice injecting recombinant LCN2 and pristane-treated LCN2+/− mice. However, examination of naive LCN2+/− mice did not reveal any abnormalities in their resting immune cell populations, indicating that LCN2 does not play an essential role in the development of the immune system. Flodorer et al. demonstrated that DC-secreted LCN2 could induce a Th1 phenotype in CD8+ T cells. However, they reported that CD4+ T cells did not express 24p3R. We then detected 24p3R expression in CD4+ T cells. Protein expression of 24p3R in CD4+ T cells was confirmed in our study. La Manna et al. has described a direct effect of LCN2 on CD4+ T cells. These previous studies supported our finding that LCN2 overexpression induced Th1 differentiation. Myeloid cells, T cells, and TECs could be the source of LCN2 in LN. However, further studies are required to assess the contribution of the individual source of LCN2 to inflammation.

STAT4 was identified as a functional target of LCN2 in this study, where it mediates the regulatory effects of LCN2 on Th1 cell differentiation. Activated STAT4 is necessary for IL-12–induced Th1 differentiation. Stat4−/− lymphocytes demonstrate a propensity toward the development of Th2 cells, producing increased levels of IL-4 and IL-10. These findings enabled us to address the fundamental question of how Th1 cell differentiation is regulated by LCN2. We demonstrated that LCN2 deficiency greatly inhibited STAT4 activation under the condition of Th1 cell differentiation. STAT4 phosphorylation in response to IL-12 is dependent on interaction with tyrosines in the cytoplasmic domain of the IL12Rβ2 subunit. We found IL12Rβ2 expression was significantly lower in LCN2−/− Th1 cells, which might be responsible for the lack of STAT4 phosphorylation. Thus, LCN2 may mediate STAT4-associated differentiation of Th1 cells in the pathogenesis of LN.

Beyond its role as a biomarker, LCN2 is actively involved in the mechanism underlying kidney diseases. In a subtotal nephrectomy-induced CKD model, LCN2 plays a key role in the progression of renal failure by mediating the mitogenic effect of the EGF receptor. In Pawar et al.’s study on antibody-mediated nephrotoxic nephritis, they proved that LCN2 promoted inflammation and apoptosis of mesangial cells. Here, we described a pivotal role of LCN2 in promoting Th1 cell differentiation. IFN-γ produced by Th1 cells leads to further activation of macrophages and aggravates the inflammatory response. Indeed, we found that removal of LCN2 inhibited the kidney inflammation in both the induced and spontaneous lupus model. However, several other studies proved that LCN2 was protective in certain kidney diseases. In chronic immune complex–mediated arthritis, LCN2 prevents severe tissue damage by facilitating tissue remodeling. Furthermore, LCN2 blocks necrosis and the release and activation of HMGB-1 in nephrotoxic serum nephritis. Therefore, the role of LCN2 in kidney diseases may depend on the particular disease context and the associated pathologic mechanisms.

In conclusion, this study provides evidence that LCN2 promotes Th1 cell differentiation through the IL-12/STAT4 pathway in an autocrine or paracrine manner, leading to the exacerbation of LN. During pathologic exacerbation of LN, the kidney (TECs and infiltrating leukocytes) can also serve as the source of LCN2, forming a positive feedback loop (Figure 7). Together, our findings show that LCN2 plays an important role in the formation of LN and immune imbalance in SLE, providing implications for disease monitoring and a potential target for SLE treatment.

**DISCLOSURES**

All authors have nothing to disclose.

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Dr. Xiaojun Tang, and Dr. Xiaohao Duan acquired the data; Dr. Weiwei Chen, Dr. Shufang Wu, and Dr. Yuemei Xu analyzed the data; Dr. Weiwei Chen, Dr. Dandan Wang, and Dr. GenHong Yao wrote the manuscript; Dr. Wanjun Chen, Dr. Xiangshan Fan, and Dr. Liwei Lu assisted in experimental design and editing of the manuscript.

SUPPLEMENTAL MATERIAL

This article contains the following supplemental material online at http://jasn.asnjournals.org/lookup/suppl/doi:10.1681 ASN/2019090937/-DCSupplemental.

Supplemental Figure 1. Expression of LCN2 in immune subsets of human PBMCs.

Supplemental Figure 2. LCN2 expression in SLE naive CD4+ T cells is positively correlated with clinical indexes.

Supplemental Figure 3. LCN2 levels increase with the progression of LN in MRL/lpr mice.

Supplemental Figure 4. Effects of recombinant LCN2 injection on MRL/lpr mice.

Supplemental Figure 5. LCN2−/− mice develop normal immune cell populations.

Supplemental Figure 6. Inflammatory cells and Th cells in pristane-treated LCN2−/− mice.

Supplemental Figure 7. Effects of anti-LCN2 antibodies on Th cells in MRL/lpr mice.

Supplemental Figure 8. Knockdown efficiency of LCN2−/− CD4+ T cells.

Supplemental Table 1. Demographic characteristics of LN patients in this study.

Supplemental Table 2. Baseline characteristics of pathologic classifications of LN patients.

Supplemental Table 3. Sequences of PCR primers.

REFERENCES

38. Ooi JD, Kitching AR: CD4+ Th1 cells are effectors in lupus nephritis--but what are their targets? Kidney Int 82: 947–949, 2012

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