Supplemental Methods for

Kidney tubular epithelial cell ferroptosis links glomerular injury to tubulointerstitial pathology in lupus nephritis.

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Supplemental Methods

Mice

Female MRL/lpr, male *FtH*$^{fl/fl}$, and parental strains of (NZW X BXSB) F1 mice were purchased from the Jackson Laboratories (Bar Harbor, USA), and *Pepck*$^{Cre/wt}$ mice were gifts from Dr. Volker Haase (Vanderbilt University). The transgenic mice deficient in *FtH1* expression only in the kidney's proximal tubules (*FtH*$^{PT-/-}$) were generated as described in our previous studies (1, 2). Animals were maintained on standard chow in SPF conditions. Tissue was collected from 4 and 16 week old (NZW X BXSB) F1 males and 8 and 20 week old female MRL/lpr mice.

Induction of nephrotoxic serum glomerulonephritis in mice

10-12 week old female *FtH*$^{PT-/-}$ and *FtHPT*$^{+/+}$ littermate control mice were preimmunized intraperitoneally with 200 µg of sheep IgG (Serotec) in Addavax (Invivogen), followed by intravenous injection of sheep nephrotoxic serum or normal donor sheep serum (2.5 µL of serum per gram of mouse, Probetex, San Antonia, Tx) 4 d later as described previously (3). Tissue was collected 14 d later.

Biochemical assays, tissue samples, and histology

Proteinuria was assessed by adding 50 µL urine on Siemens Multistix 8 SG dipsticks. Ketamine (120 mg/kg)/xylazine (12 mg/kg) mix was used to anesthetize the mice. Blood was drawn from the axillary vein before euthanasia. Kidney slices were processesd for gene, protein and histology as described (4). Paraffin-embedded or cryosections of human biopsies or mouse kidney sections (3 µm) were used for staining purposes.

Cell Culture, siRNA knockdown and human pateints serum studies

HK-2 cells were maintained in ATCC recommended keratinocyte serum-free medium with the addition of bovine pituitary extract (0.05 mg/mL) and human recombinant epidermal growth factor (5 ng/mL). For gene knockdown studies, silencer select siRNA for human FtH1 and negative control siRNA (Catalog number: 4392421 and 4390843 respectively, ThermoFisher Scientific) were used. HK-2 cells (2 X 10^5/well) were transfected with the FtH1 and negative control siRNA, respectively, using Opti-MEM reduced serum medium and Lipofectamine RNAiMAX Transfection Reagent (ThermoFisher Scientific) in 12 well plates as per the

manufacturer's instructions. Gene knockdown efficiency was measured after 52 hrs by RT-PCR. At this time point medium was changed and cells were incubated for 12 hrs with vehicle, 200 μM ferrous sulfate, 20 mg/mL human albumin or a mixture of 200 μM ferrous sulfate and 20 mg/mL human albumin. Lactate dehydrogenase (LDH) content in the cell culture supernatant was used to measure cell death (Cayman Chemical). All experiments were carried out in a medium containing 5% serum from lupus nephritis (Class IV) patients or healthy donors. Markers of ferroptosis (ACSL4 and GPX4) were measured by Western blotting. Liproxstain-2, a proprietary new generation ferroptosis inhibitor by ROSCUE Therapeutics was used. To evaluate the efficacy of Liproxstatin-2 (ferroptosis inhibitor), 2 x 10^5 cells were pretreated with 100 ng/mL or DMSO for 12 hrs before serum treatment. In some experiments liproxstain-2 was added after 1 or 4 hours after the addition of patient serum. All data were analyzed 24 hrs after addition of patient or healthy donor serum.

Immunohistochemistry

Paraffin-embedded human biopsies or mouse kidney sections (3 μm) were stained for acyl-CoA synthetase long-chain family member 4 (ACSL4) or 4-hydroxynonenal (4-HNE) using standard protocols. Sections were deparaffinized in xylenes, rehydrated in a series of ethanol rinses (100%–70%), and washed in distilled water. Sections were then incubated in 3% H_2O_2 in methanol for 20 minutes. After antigen retrieval by heating in citrate buffer and treating with avidin and biotin for 15 minutes each (Avidin/Biotin Blocking Kit; Vector Laboratories), the sections were incubated in blocking buffer containing 10% donkey serum in 0.1 M sodium phosphate buffer pH 7.4 (phosphate buffer) at room temperature for 30 minutes. Next, sections were incubated with anti-ACSL4 (1: 500, in 1% BSA/phosphate buffer; sc-271800, Santa Cruz) or 4-HNE antibody (1:1000 in 1% BSA/phosphate buffer; Abcam, Boston, MA) overnight at 4°C. After three washes with phosphate buffer for 5 minutes each, sections were incubated with biotinylated donkey anti-mouse or donkey anti-goat secondary antibody (1:400 dilution; Vector Laboratories) for 1 hour. After three washes in phosphate buffer, sections were incubated with ABC ready-to-use reagent (VECTASTAIN Elite ABC Kit; Vector Laboratories) for 30 minutes. After another three washes with phosphate buffer, the sections were incubated with 3,3′-diaminobenzidine for 5 minutes, followed by washing with distilled water. Finally, the sections were counterstained with 1% methylene blue solution, washed in water, and dehydrated with xylene. Sections were imaged using a Nikon microscope.

Immunofluorescence and detection of immune complex.

Three to five micron cryostat-cut kidney sections were used for the immunofluorescence detection of α smooth muscle actin (αSMA), F4/80+ macrophages, CD4+ T cells and immune complexes. Detailed method is provided in the supplemental methods sections. Briefly, the kidney sections were air-dried and incubated in PBS with 0.3% TritonX-100/10% horse serum. After washing with PBS, FC receptors were blocked with anti-CD16/32 (2.4g2, ThermoFisher, Waltham, MA USA) antibody. This was followed by incubation with FITC-labeled anti-αSMA (1A4, 1:30; Sigma, St. Louis, MO), PE-labeled F4/80 (BM8, 1:20; ThermoFisher), PE-labeled CD4 (GK1.5, 1:20; ThermoFisher) in 10% horse serum/PBS for 1.5 hrs. The sections were then washed in PBS and mounted with ProLong Gold antifade agent with DAPI (ThermoFisher).
To examine glomerular immune complex deposits, 5 mm snap-frozen kidney sections were fixed in acetone, washed in PBS, incubated in 3 % hydrogen peroxide for 10 min, and rinsed in PBS. Slides were incubated in FITC–conjugated monoclonal anti-IgG antibody (Santa Cruz, Dallas, Tx) at a 1:50 dilution in PBS for 1 hr at room temperature. Slides were then rinsed in PBS and mounted with a coverslip using Vectashield hard set medium (Vector H-1400, Vector Laboratories, Burlingame, CA). Fluorescence was detected using a Nikon microscope.

Detection of serum IgG, IgG2a, and anti-dsDNA antibodies.

Mouse serum IgG and IgG2a were detected using commercial ELISA kits (ThermoFisher) as per manufacturers' instructions. Anti-dsDNA IgG was measured in 1:100 diluted serum using plates coated with 50 μg/ml dsDNA as described (5) .

Preparation of Kidney and Cell Extracts, and Western Blotting

Western blotting was performed as described in (4). Cells (HK-2) or snap-frozen tissue sections were homogenized in Tris-Triton tissue lysis buffer containing complete protease inhibitor cocktail (Halt Protease and Phosphatase Inhibitor Cocktail; Thermo Fisher Scientific, Rockford, IL) using a Dounce Homogenizer. GPX4 and ACSL4 expression were measured in whole lysates. Protein content in the homogenate was estimated using the Pierce BCA protein estimation kit (Thermo Fisher Scientific). Ten micrograms of protein per sample were loaded on a 4-12% NuPage Bis-Tris gel under reducing conditions. The resolved proteins were transferred onto a nitrocellulose membrane (LI-COR Biotechnology, Lincoln, NE) and probed with rabbit

monoclonal anti-GPX4 (1:1000, ab125066, Abcam), mouse monoclonal anti-ACSL4 (1:100, sc-271800, Santa Cruz), rabbit monoclonal anti-FtH1 (1:1000, ab183781, Abcam) and mouse monoclonal anti-βactin (1:7000, ab8226, Abcam) antibodies. The primary antibodies were detected using donkey anti-rabbit Alexa 800 and donkey anti-mouse Alexa 800 antibodies (LI-COR). Mouse monoclonal anti-β-actin (Abcam) was used as the loading control and detected using donkey anti-mouse Alexa 680 antibody (LI-COR). Data were quantified using densitometry software (LI-COR).

RT-PCR

RNA isolation, cDNA synthesis and RT-PCR was performed as described in our previous publication (4). For RNA isolation, frozen tissues or live cells were resuspended in RLT buffer (Qiagen, Valencia, CA) and homogenized using the TissueLyser system or Qiashredder (Qiagen). RT was performed as described in our previous publication(4)**.** Total RNA from homogenates was purified using the RNAeasy mini kit (Qiagen) following the manufacturer's instructions. Then, 0.2-1 µg of RNA was used to synthesize cDNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). The cDNA template was mixed with iTAQ SYBR green universal supermix (Bio-Rad), and quantitative PCR was performed on a CFX Connect system (Bio-Rad). Human or mouse predesigned gene primers were purchased from Bio-Rad. Human or mouse *PPIA* was amplified in parallel and used as the reference gene in quantification. Data are expressed as relative gene expression and were calculated using the 2^[−ΔC(T)] method.

Preparation of membrane fractions and liquid chromatography-mass spectrometric analysis of renal lipids

Lipids were extracted with a modified Bligh-Dyer protocol (6). For each animal, 10 mg kidney tissue was washed with PBS before being subject to homogenization with an Omni TH homogenizer (Warrenton, VA) in tissue protein extraction reagent (TPER) (Thermo Fisher Scientific; Waltham, Massachusetts) supplemented with Halt protease and phosphatase inhibitors (Thermo Fisher Scientific). The lysate was incubated on ice for 30 minutes while vortexing every 10 minutes. The lysates were then subject to centrifugation at 13,000 rpm for 10 minutes using a tabletop centrifuge (Thermo IEC) before the supernatant was subject to ultracentrifugation for 30 minutes at 34,000 rpm at 4º C using an SW55.1 rotor and Optima L-90K ultracentrifuge (Beckman Coulter; Schaumburg, IL). The pellet was reconstituted in TPER and then sonicated twice for 5-second intervals. A BCA assay was performed to determine protein concentration.

Lipids were extracted with a modified Bligh-Dyer protocol(6). Two milliliters of methanol, 800 uL of dichloromethane, 20 uL of lipid membranes, 980 ul of water, and 50 ul of 1:5 diluted EquiSplash Lipidomix heavy isotope Standards (consisting of 20 ug/L each of a mixture of 13 deuterated lipids, Avanti Polar Lipids, Inc., AL, USA) were combined in 13x100 mm glass screw-cap tubes. The mixture was left at room temperature for thirty minutes. 1 mL water and 0.9 mL methylene chloride were added and tubes were gently inverted 10 times and centrifuged at 200 g for 10 minutes. After centrifugation, the lower layer was collected. The samples were extracted with 800 ul of dichloromethane, vortexed, centrifuged before collecting the lower layer. Finally, the organic phase was dried under nitrogen gas and reconstituted in 50 uL of methanol. Targeted liquid chromatography-mass spectrometric analysis was performed on a QTRAP 6500+ triple quadrupole mass spectrometer (AB SCIEX) and a Nexera liquid chromatography system (Shimadzu). The column used was a Phenomenex luna 3µm 2.1x100 mm column with mobile phase A 1 mM ammonium acetate in 7/93 dichloromethane/acetonitrile and mobile phase B consisting of 1 mM ammonium acetate in 50/50 water/acetonitrile pH 8.2. The gradient was 17.5 minutes long with a 5 ul injection volume. About 1,200 lipids were detected. Differentially regulated lipids of different classes were compared between non-nephritic and nephritic kidneys, and $p < 0.05$ was considered significant. A heatmap was used to visualize the results.

Statistics

Statistical significance was determined by applying a Mann-Whitney test for groups not passing the normality test. 1-way and 2-way analysis of variance (ANOVA) with Tukey`s or Holm-Šídák's multiple comparisons test were used to compare more than 2 groups of experimental conditions and represented as mean ± SEM. All analyses were performed using GraphPad Prism 9 (GraphPad Inc, San Diego, CA).

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