Renal Glycosphingolipid Metabolism Is Dysfunctional in Lupus Nephritis

Tamara K. Nowling,*† Andrew R. Mather,† Thirumagal Thiagarajan,† María José Hernández-Corbacho,§ Thomas W. Powers,‖ E. Ellen Jones,‖ Ashley J. Snider,§¶ Jim C. Oates,*† Richard R. Drake,‖ and Leah J. Siskind**

*Research Service, Ralph H. Johnson Veterans Affairs Medical Center, Charleston, South Carolina; †Department of Medicine, Division of Rheumatology and Immunology and Departments of ‡Drug Discovery and Biomedical Sciences and †Cell and Molecular Pharmacology, Medical University of South Carolina, Charleston, South Carolina; §Department of Medicine, Stony Brook University Medical Center, Stony Brook, New York; ‖Research Service, Northport Veterans Affairs Medical Center, Northport, New York; and **Department of Pharmacology and Toxicology and the James Graham Brown Cancer Center, University of Louisville, Louisville, Kentucky

ABSTRACT

Nearly one half of patients with lupus develop glomerulonephritis (GN), which often leads to renal failure. Although nephritis is diagnosed by the presence of proteinuria, the pathology of nephritis can fall into one of five classes defined by different forms of tissue injury, and the mechanisms involved in pathogenesis are not completely understood. Glycosphingolipids are abundant in the kidney, have roles in many cellular functions, and were shown to be involved in other renal diseases. Here, we show dysfunctional glycosphingolipid metabolism in patients with lupus nephritis and MRL/lpr lupus mice. Specifically, we found that glucosylceramide (GlcCer) and lactosylceramide (LacCer) levels are significantly higher in the kidneys of nephritic MRL/lpr lupus mice than the kidneys of non-nephritic lupus mice or healthy controls. This elevation may be, in part, caused by altered transcriptional regulation and/or activity of LacCer synthase (GalT5) and neuraminidase 1, enzymes that mediate glycosphingolipid metabolism. We show increased neuraminidase 1 activity early during the progression of nephritis (before significant elevation of GlcCer and LacCer in the kidney). Elevated levels of urinary LacCer were detected before proteinuria in lupus mice. Notably, LacCer levels were higher in the urine and kidneys of patients with lupus and nephritis than patients with lupus without nephritis or healthy controls. Together, these results show early and significant dysfunction of the glycosphingolipid metabolic pathway in the kidneys of lupus mice and patients with lupus nephritis and suggest that molecules in this pathway may serve as early markers in lupus nephritis.


SLE is a heterogeneous autoimmune inflammatory disease presenting in varying degrees and symptoms and affecting many organs, including skin, joint, respiratory, cardiac, and kidney.1–4 SLE is thought to result from environmental triggers and predisposing genetic factors.2–5 SLE broadly encompasses accumulation and subsequent deposition of immune complexes in or binding of autoantibodies to tissues, causing injury and further activating an immune response.4,6,7 Approximately one half of patients with SLE develop renal disease.1,8 Lupus nephritis (LN) is part of a larger family of GN diseases that often leads to end stage kidney disease.9 Although LN varies from proliferative, inflammatory lesions to bland, membranous lesions, it is diagnosed by the presence of proteinuria or nephritic urine.10

Received May 22, 2014. Accepted August 7, 2014.
Published online ahead of print. Publication date available at www.jasn.org.

Correspondence: Dr. Tamara K. Nowling, Department of Medicine, Medical University of South Carolina, 96 Jonathan Lucas Street, CSB 912 MSC 637, Charleston, SC 29425-6370, or Dr. Leah J. Siskind, Department of Pharmacology and Toxicology, University of Louisville, 505 South Hancock Street, CTRB 203, Louisville, KY 40202. Email: Nowling@musc.edu or leah.siskind@louisville.edu.

Copyright © 2015 by the American Society of Nephrology
Glycosphingolipids are a heterogeneous class of lipids in the sphingolipid family that plays a role in the regulation of cellular processes, such as proliferation and inflammation. Highly abundant in the kidney, glycosphingolipids play a role in a variety of kidney diseases, including GN, kidney cancer, nephropathy, and polycystic kidney disease. Hexosylceramide (HexCer), glucosylceramide (GlcCer), and galactosylceramide (GalCer) are generated by the addition of sugar moieties to ceramide. GlcCer is generated from GlcCer synthase-mediated addition of glucose to ceramide (Figure 1). After formed, lactosylceramide (LacCer) synthases catalyze the addition of galactose to form LacCer. Complex glycosphingolipids are formed by further additions to LacCer. For example, gangliosides are formed by addition of sialic acid residues to LacCer. After formed, glycosphingolipids are transported within in the cell to the plasma membrane and lysosome. Sialidases (neuraminidases [NEUs]) remove sialic acids from gangliosides and proteins. NEUs are localized to the lysosome (NEU1), cytosol (NEU2), plasma membrane (NEU3), and mitochondria (NEU 4), and NEU1 can be secreted extracellularly to act on the plasma membrane. Thus, LacCer can be regenerated by NEUs breaking down gangliosides.

Aberrant glycosphingolipid metabolism occurs in several autoimmune diseases, including autoimmune diabetes, thyroid disease, and various neuropathies. Thus, we hypothesized a role for glycosphingolipids in LN, a prototypical autoimmune kidney disease. We tested this hypothesis in the MRL/MpJ-Fas−/− (MRL/lpr) mouse model of LN and human kidney biopsies and urine samples. We show that renal glycosphingolipid metabolism is dysregulated in lupus-prone mice, resulting in elevated levels of renal and urinary glycosphingolipids. Glycosphingolipid accumulation is, at least in part, caused by transcriptional upregulation of NEU1, with significant increase in NEU activity early in disease that continues to increase as disease progresses. Of translational importance, urinary LacCer levels and NEU1 protein levels were significantly higher in patients with LN than patients with lupus without nephritis and healthy subjects. LacCer levels were also increased in kidney biopsies of patients with LN compared with controls. To our knowledge, this is the first report of glycosphingolipid dysfunction in LN, and data strongly suggest that glycosphingolipids may serve as early markers of LN.

RESULTS

Kidney Glycosphingolipids Are Elevated in Nephritic MRL/lpr Lupus Mice

Sixteen-week-old MRL/lpr lupus mice, a model for classes 3 and 4 LN, have nephritis characterized by renal immune complex deposition, lymphocyte infiltration, proteinuria, and significant renal pathology compared with age-matched MRL/MpJ lupus-prone mice, which develop nephritis much later in life. We analyzed renal cortical glycosphingolipid levels in MRL/lpr and MRL/MpJ mice and found that HexCers and LacCers were 7- to 8-fold higher in 16-week-old MRL/lpr mice compared with non-nephritic age-matched MRL/MpJ mice (Figure 2). The increases in HexCers and LacCers are likely not because of an increase in ceramides, because only C24-ceramide was significantly elevated (Figure 3A). HexCers consist of both GlcCer and GalCer, which cannot be distinguished by HPLC-mass spectrometry (MS)/MS. Supercritical fluid tandem MS was performed at the Medical University of South Carolina Lipidomics Core Facility to separate GlcCer and GalCer. Most of the GlcCer species are significantly elevated (Figure 3B), whereas GalCer species remained unchanged (Figure 3C), indicating that HexCer levels can be considered representative of GlcCer levels. All LacCer species were elevated in the kidney cortices of MRL/lpr compared with MRL/MpJ mice, with C16- and C24:1-LacCers significantly elevated (Figure 3D). Matrix-assisted laser desorption/ ionization imaging MS (MALDI-IMS) was used to determine the tissue distributions of HexCer (Figure 3E) and LacCer (Figure 3F) in the kidneys of 18-week-old nephritic MRL/lpr mice compared with age-matched healthy C57BL/6 mice with representative MS/MS spectra (Figure 3G). These results confirm that GSLs are elevated compared with normal healthy controls and show that different species have different tissue distributions, with most being elevated in the cortex region, where many of the pathologic changes occur in LN.
Kidney Glycosphingolipid Degradation Is Dysregulated in Nephritic MRL/lpr Lupus Mice

GlCers and LacCers can accumulate by increased synthesis from ceramides and/or increased degradation of complex glycosphingolipids. To determine the mechanism of kidney GlcCer and LacCer accumulation in MRL/lpr mice, kidney cortex message levels of enzymes that directly regulate glycosphingolipid metabolism were determined. Message levels of GlCer synthase, which synthesizes GlcCer from ceramide, and glucocerebrosidase, which mediates the breakdown of GlcCer to ceramide, were unchanged in nephritic MRL/lpr compared with non-nephritic MRL/MpJ mice (Figure 4, A and B). Of three major LacCer synthases (GalT5, GalT6, and GalT7) that can synthesize LacCer from GlcCer, only GalT5 message was altered with lower expression in nephritic compared with non-nephritic mice (Figure 4C). Additional enzymes that mediate glycosphingolipid metabolism include galactosidase (GLA), which breaks down globotriaosylceramide to LacCer, GM3 synthase, which synthesizes gangliosides from LacCer, and Neus, which break down gangliosides to form LacCer. GLA and GM3 synthase levels were not significantly changed (Figure 4, D and E), whereas Neu1 was significantly elevated (Figure 4F) in MRL/lpr compared with MRL/MpJ mice. Results in Figures 3 and 4 are summarized in the pathway diagram in Figure 1.

Increased Neu1 Message Translates to Increased Renal Cortical NEU Activity in Lupus Mice

NEU activity was significantly higher in nephritic MRL/lpr mice compared with non-nephritic MRL/MpJ mice (Figure 5A). We wanted to determine the mechanism responsible for increased Neu1 message. Sterol regulatory element-binding proteins (SREBPs) are transcription factors known to regulate global lipid metabolism. They can be activated by glycosphingolipids and induce expression of genes that regulate glycosphingolipid synthesis, including Neu1.21 SREBPs are associated with several kidney diseases,22 but their involvement in LN has not been shown. Renal cortical SREBP-1 protein levels were increased in MRL/lpr mice compared with MRL/MpJ control mice (Figure 5B). Both SREBP-1a and SREBP-1c were shown to upregulate Neu1 in myotubes.21 To determine which SREBP-1 isoform may be regulating Neu1 in the lupus kidneys, we measured expression of factors in cholesterol and fatty acid metabolism, pathways regulated by SREBP-1a and SREBP-1c, respectively.22 Fatty acid synthase message levels (Figure 5C) and triglyceride levels (Figure 5D) were significantly increased, whereas 3-hydroxy-3-methyl-glutaryl-CoA reductase message levels (Figure 5E) and cholesterol levels (Figure 5F) were not significantly different in the renal cortices of MRL/lpr compared with MRL/MpJ mice. These results are consistent with an upregulation of SREBP-1c but not SREBP-1a. Together, these results suggest that accumulation of GlcCer and LacCer in LN occurs, in part, by SREBP-1c induction of Neu1 expression, which breaks down gangliosides to yield LacCers.

Renal Glycosphingolipid Dysfunction Begins Early in Disease and Corresponds with Elevated Urinary LacCer

Renal injury typically results in excretion of plasma proteins into the urine, with albumin accounting for the majority of urine proteins during severe glomerular injury. In the MRL/lpr model, urinary albumin is elevated between 12 and 24 weeks of age.10 To determine if glycosphingolipid levels are detectable in the urine of lupus mice and how early in disease they are elevated with respect to albuminuria, urinary albumin and C16-LacCers (the most abundant species in the urine of mice; data not shown) were quantified in 24-hour urine collections from age-matched MRL/lpr and MRL/MpJ mice. As reported previously,23 proteinuria increased at 16–18 weeks, with significant differences at 20 weeks (Figure 6A). C16-LacCers increased at 14–16 weeks, with significant differences at 18 weeks (Figure 6B). These results suggest that C16-LacCers increase before proteinuria and may be early markers of nephritis.

We then measured renal cortex GlcCer/LacCer levels and NEU activity earlier in disease to determine when they begin to increase. There was a slight increase in the major GlcCer and LacCer species between 11 and 14 weeks (Figure 6, C and D), which significantly increased from 14 to 18 weeks of age (Figure 6, C and D). These changes paralleled increases in urinary C16-LacCer (Figure 6B). NEU activity in renal cortices of the same mice was significantly higher in 11-week-old MRL/lpr mice compared with the activity in 18-week-old healthy C57BL/6 mice, increasing over time (Figure 6E). NEU activity increased significantly in MRL/lpr kidneys between 11 and 18 weeks of age and between 14 and 18 weeks of age (Figure 6E).
These results indicate that NEU activity becomes significantly elevated before accumulation of GlcCer and LacCer and suggest that dysfunctional glycosphingolipid metabolism occurs very early in disease in the MRL/lpr kidney.

Urinary and Kidney LacCer Levels Are Elevated in Patients with LN

To determine whether glycosphingolipid metabolism is dysfunctional in patients with LN, urinary C16-LacCer levels were measured and normalized to creatinine or eGFR to account for differences in renal function. When normalizing to either creatinine or eGFR, C16-LacCer was significantly elevated in the urine of patients with LN compared with patients with lupus without nephritis and healthy control subjects (Figure 7A) (data not shown), whereas levels in the serum were not significantly different among the groups (Figure 7B). Although the sample size was relatively small, the significant differences in the urine were large, emphasizing that GSL metabolism in the kidney may be important in the pathogenesis of nephritis. We then analyzed LacCer expression in renal biopsies from patients with LN compared with controls. A low level of expression throughout the tissue of the control biopsies (Figure 7C, upper panel) was observed as expected, because glycosphingolipid expression is abundant in normal, healthy kidneys. However, we observed a general increase in LacCer staining outside of the glomeruli (Figure 7D, upper panel) and intense staining within the glomeruli (Figure 7E, upper panel) in the biopsies of LN patients. The intense staining corresponds with the increase in cellular expansion observed in LN (Figure 7, D and E compared with Figure 7C, upper panel). Confocal imaging at higher power shows that the intense staining in the glomeruli in Figure 7E is located in the mesangial region (Figure 7F). We then measured NEU activity and NEU1 protein levels in urine samples from the same patients with lupus analyzed in Figure 7A. We observed measurable NEU activity in only two samples: both patients with class 4 nephritis (data not shown). The lack of measurable NEU activity in most of the samples may be because of the presence of an inhibitor of NEU activity in the urine and/or extended handling of the samples. NEU activity is extremely sensitive to temperature changes. NEU1 protein levels in these same urine samples were increased in patients with LN compared with patients with lupus without nephritis and healthy controls (Figure 7G) (data not shown). The results in Figure 7G are representative of eight samples analyzed for each group, with all eight patients with LN expressing measurable levels of NEU1 protein. Compared with patients with LN, light bands were observed in three patients with lupus without nephritis and one healthy control. These results show that renal glycosphingolipid dysfunction occurs in patients with LN.
LN and that elevated levels of LacCer and NEU1 protein in the urine of patients with LN likely are cause by renal and not systemic GSL dysfunction. Coupled with results in mice, these results suggest that urinary LacCer may serve as an early marker for nephritis and/or a screening tool to identify individuals likely to develop nephritis.

**DISCUSSION**

To our knowledge, this is the first report showing glycosphingolipid metabolic dysfunction in the kidneys of mice and patients with LN. Our studies used the MRL/lpr lupus-prone mouse model and the lupus-prone mouse model from which they were derived, the MRL/MpJ strain. We compared age-matched nephritic MRL/lpr with non-nephritic MRL/MpJ mice. We also used the nonautoimmune-prone C57Bl/6 strain as a healthy control. Both MRL strains develop lupus similar to that observed in human lupus pathologies, including increased IgG levels, autoantibody production, proteinuria, and eventually, development of end stage kidney disease, with the lpr mice developing disease at an accelerated rate because of a mutation of the Fas gene.24 We showed increased kidney HexCer and LacCer in nephritic MRL/lpr mice compared with non-nephritic MRL/MpJ mice. Similarly, urinary LacCers in nephritic mice were significantly elevated compared with non-nephritic mice. Moreover, the elevation of LacCer in the urine (16 weeks) occurred before development of significant proteinuria (18 weeks), suggesting that urinary LacCer may be an earlier or more sensitive marker of nephritis.

By measuring expression of the enzymes involved glycosphingolipid metabolism, we identified possible mechanisms for accumulation of renal GlcCer and LacCer in nephritic mice. The only significant difference in expression of enzymes in the synthesis pathway was for GaT5, one of the LacCer synthase genes, which was significantly downregulated in nephritic mice. A lack of GaT5 was shown to induce accumulation of GlcCer.25 Therefore, accumulation of renal GlcCer in LN mice may be, in part, because of decreased GaT5 expression.

Neu1 expression and NEU activity were significantly elevated in lupus mice with nephritis compared with non-nephritic lupus mice. The enzymatic assay does not distinguish between NEU isoforms, and thus, we cannot determine the contribution of NEU1 to the overall increase in NEU activity. If increased NEU activity is a major contributor to the elevation of LacCer and/or GlcCer, the increase in activity should precede increased GlcCer and LacCers. In MRL/lpr mice, immune complex deposition in the kidney begins around 8–10 weeks of age followed by local renal production of MCP-1 and other cytokines and renal IgG and C3 deposition with immune cell infiltration occurring between 14 and 16 weeks of age.10,26 NEU activity was significantly increased earlier in disease than the increase in GlcCer and LacCers. The early increase in NEU activity in the kidney may be a response to immune complex deposition and local expression of cytokines. This dysregulation may then contribute to the progression of nephritis by promoting additional local renal cytokine production and leukocyte infiltration. Indeed, exogenously added LacCer and GlcCer to mesangial cells induce production of several cytokines/chemokines important in leukocyte infiltration in LN (J. Siskind, unpublished observations). Moreover, the timing of immune cell infiltration in the MRL/lpr kidney corresponds with the significant increase in GlcCer and LacCer species from 14 to 18 weeks of age. Our previous studies in the MRL/lpr model showed that mice with improved disease have significantly decreased levels of the major GlcCer/LacCer species in their T cells at 18 weeks of age.27 We speculate that infiltrating T cells and other immune cells may contribute to the increased glycosphingolipid levels in the kidney either directly through their expression of GlcCer and LacCer and/or
indirectly by activating resident renal cells that further elevate GlcCer and LacCer levels. Our results here analyzing the human serum and urine samples suggest that the elevated lipids are largely caused by renal-specific rather than systemic contributions. Future studies will define mechanisms responsible for the significant increases in glycosphingolipid metabolism with respect to the pathologic hallmarks of nephritis development.

SREBP-1 is a transcription factor that regulates expression of genes involved in lipid homeostasis. When the mature nuclear forms of SREBP-1a and SREBP-1c were overexpressed in human skeletal muscle cells, microarray analysis revealed increased expression of several genes involved in glycosphingolipid metabolism, including Neu1.21 We showed an increase in the mature fragment of SREBP-1c in the kidneys of nephritic mice; because established targets of SREBP-1c are also upregulated in the kidneys of LN-prone mice, data indicate that the mature fragment of SREBP-1c is, indeed, active. Elevated levels of GlcCers and LacCers are sufficient to induce SREBP activation.28 Thus, glycosphingolipids may be up- and downstream of SREBP activation. Aberrant activation of SREBPs and renal lipid accumulation occur in kidney diseases and were linked to increased production of inflammatory cytokines and glomerulosclerosis.29,30 Overexpressing the active form of SREBP-1 in the kidneys of mice induces proteinuria.31

Our results suggest, for the first time, that activation of SREBPs and accumulation of lipids within the kidney also occur in LN.

Of translational significance, data showed that patients with LN exhibited dysfunction of glycosphingolipid metabolism similar to the MRL/lpr nephritic mice. Patients with LN had elevated levels of urinary LacCer and NEU1 but not serum LacCer compared with patients without nephritis and healthy controls. LacCer staining of renal biopsies from patients with LN showed intense staining around the glomeruli in the mesangial region compared with biopsies from control patients. Together, our results suggest that the elevated urinary LacCer levels in patients with LN are caused by GSL dysfunction in the kidney. Interestingly, the levels of urinary NEU1 seemed to be higher in patients with class 4 compared with class 3 nephritis. Future work will be aimed at determining if there are class-specific differences in urinary and/or renal NEU1 and LacCer levels.

Together, our results indicate that GlcCers and LacCers are elevated in a mouse model of LN as well as patients with LN. Our data suggest that increased NEU1 activity may be the mechanism by which these lipids accumulate. Data indicate that NEU1 is upregulated at the transcriptional level and suggest that SREBP-1c may play a role in NEU1 up-regulation and dysfunctional glycosphingolipid metabolism in LN mice. Data suggest that glycosphingolipid metabolism is dysfunctional early in disease before renal pathogenesis. Because GlcCers and LacCers are present in the urine, they also represent potential biomarkers for diagnosis of LN and may be a noninvasive means for distinguishing different classes of nephritis. Future studies are aimed at analyzing longitudinal samples of patients without nephritis who subsequently developed nephritis to determine if LacCer may serve as an early marker for nephritis and using inhibitors of glycosphingolipid metabolism in lupus-prone mice to determine if glycosphingolipids are, indeed, mediators of disease.

**CONCISE METHODS**

**Patient Renal Biopsies and Urine Samples**

Clean catch urine samples for the LacCer and NEU protein level analyses were obtained from frozen, centrifuged, and aliquoted samples that were collected for an unrelated lupus urine biomarker study.32 All volunteers gave documented informed consent to participate in Institutional Review Board-approved protocols. Analyzed samples were from patients (predominantly African Americans) meeting American College of Rheumatology revised criteria for...
SLE without nephritis, subjects with active LN at entry, and control subjects without autoimmune or inflammatory disease. Spot urine and blood collections and clinical evaluations were performed at entry. Patient demographics and clinical measures ascertained from medical records for subjects (urine samples [Figure 7A] and serum samples [Figure 7B]) are shown in Table 1. Disease activity as measured by the Systemic Lupus Erythematosus Disease Activity Index, eGFR, and the National Institutes of Health morphologic indices of renal biopsies (Activity Index and Chronicity Index), which correlate with clinical parameters, are included. Subjects with LN had ISN/RPS active class 2, 3, 4, or 5 nephritis by biopsy. Snap-frozen renal cortical biopsy tissue used for clinical immunohistochemistry was stored at $-80^\circ$C and later retrieved for research immunostaining as described below. Subjects did not have an active infection, ongoing pregnancy, or $>50\%$ of glomeruli with sclerosis on biopsy. Patients with lupus non-nephritis met four American College of Rheumatology criteria for lupus but had $<100$ mg protein/g creatinine in the urine and did not develop nephritis within 1 year of urine collection. Normal controls were free of known renal disease or inflammatory autoimmune disease but did not have renal biopsies performed. For the LacCer immunohistochemistry analyses, frozen OCT-embedded renal biopsies were obtained from some of the patients with LN indicated above and patients who underwent renal transplants (control) without signs of rejection. Renal biopsies were graded by the World Health Organization criteria, and those patients with classes 3 and 4 were deemed to have proliferative LN. Recipients of renal transplant met all of the entry criteria for patients with LN except for the lupus criteria and had no clinical or biopsy evidence of rejection. All samples were processed using standard quality control measures.

**Animals**

C57BL/6 and MRL/MpJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). MRL/MpJ-Fas$^{-/}$ (MRL/lpr) mice were bred in house starting with breeders purchased from The

---

**Figure 6.** Renal glycosphingolipid metabolism is dysregulated early during disease development. Timed urine collection was performed every other week in MRL/lpr and MRL/MpJ mice. (A) Albumin and (B) C16-LacCer were measured. Kidneys were collected from 11-, 14-, and 18-week-old MRL/lpr mice and 18-week-old C57BL/6 (B6) mice. (C) GlcCer and (D) LacCer levels and (E) NEU activity were measured in renal cortices. Data are means $\pm$ SDs. *$P<0.05$; **$P<0.01$; ***$P<0.001$; ****$P<0.0001$ as determined by a two-way ANOVA adjusting for multiple comparisons.
Jackson Laboratory. To avoid genetic drift in the MRL/lpr population, additional MRL/lpr mice were purchased from The Jackson Laboratory and used every other generation for breeding. Measures of disease were performed routinely on our in-house MRL/lpr colony, and they were similar, as was survival, to those published previously.10,35,36

MRL/lpr mice were used at 11–18 weeks of age as indicated. MRL/MpJ mice were used at 16 weeks of age. C57BL/6 mice were used at 18 weeks of age. All mice were maintained on a 12-hour light/12-hour dark cycle and provided food and water ad libitum. Animals were maintained under standard laboratory conditions. All animal procedures were approved by the Veterans Administration Institutional Animal Care and Use Committee and followed the guidelines of the American Veterinary Medical Association.

Kidney Homogenates
Homogenates were made from kidneys snap frozen in liquid nitrogen. The cortex was cut from the kidney and homogenized using a Tissue-Tearor (Biospec Products, Inc., Bartlesville, OK) in 20 mM Tris-HCl buffer containing a Complete Protease Inhibitor Cocktail Tablet (Roche, Indianapolis, IN). Protein concentrations were measured using the Pierce Biotech Micro BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA).

Lipid Levels
Sphingolipids were measured quantitatively in 2 mg kidney cortex homogenates or a 300-μl aliquot obtained from 24-hour urine collections in mice or spot urine collections from human subjects. Quantitation of sphingolipid species was performed by the Lipidomics Core at Medical University of South Carolina on a Thermo Finnigan TSQ 7000 Triple-Stage Quadrupole Mass Spectrometer operating in a Multiple Reaction Monitoring (MRM)-positive ionization mode as described.37,38 ESI/MS/MS analyses of endogenous GlcCer and GalCer molecular species (C18-Sphingoid Base) were performed on a Thermo Fisher TSQ Quantum Triple Quadrupole Mass Spectrometer operating in an MRM-positive ionization mode using a modified version.38 Briefly, cell pellets corresponding to about 1–3×10⁶ cells were fortified with synthetic and not natural internal standards (ISs: C8-GluCer, C8-GalCer, C12-GluCer, and C12-GalCer; Avanti Polar Lipids, Inc., Alabaster, AL) and extracted with an ethyl acetate/isopropanol/water (60/30/10 vol/vol) solvent system. After evaporation and reconstitution in 100 μl methanol, samples were injected on the Supercritical Fluid Chromatography/Mass Spectrometry Berger Minigram/TSQ Quantum SFC/MS System and gradient eluted from a Berger Silica 60 A 6-μm particle, 4.6×250-mm column with a 1.0 mM methanolic ammonium formate/2 mM aqueous ammonium formate mobile phase system. Peaks corresponding to the

Figure 7. Urine and renal LacCer is increased in patients with lupus and nephritis. (A) Urine and (B) serum from patients with active proliferative LN before induction therapy (SLE with nephritis), patients with SLE without nephritis (SLE), and healthy controls were analyzed for C16-LacCer. Data are means±SDs. ***P<0.001 compared with healthy controls and P<0.01 compared with SLE as determined by a one-way ANOVA and adjusting for multiple comparisons. LacCer staining in renal biopsies from (C) a control subject, (D) a patient with lupus and class 3 nephritis, and (E) a patient with lupus and class 4 nephritis. Upper panels are LacCer (FITC) staining, and lower panels are 4′,6-diamidino-2-phenylindole staining at ×20. Images are representative of the five control, three class 4, and two class 3 samples analyzed. (F) Confocal image of the sample in E at ×63 with FITC, DAPI, and bright-field overlay showing intense LacCer staining in the mesangial region. (G) NEU1 protein expression in urine from patients with lupus and nephritis, patients without nephritis (SLE), and healthy controls.
target analytes and ISs were collected and processed using the Xcalibur software system.

Quantitative analysis was on the basis of the calibration curves generated by spiking an artificial matrix with the known amounts of the target analyte synthetic standards and an equal amount of the ISs. The target analyte/IS peak areas ratios were plotted against analyte concentration. The target analyte/IS peak area ratios from the samples were similarly normalized to their respective ISs and compared with the calibration curves using a linear regression model.

The calibration standards (C16-GlcCer, C16-GalCer, C18:1-GlcCer, C18:1-GalCer, C24:1-GlcCer, C24:1-GalCer) were synthesized in the Lipidomics Share Resources, Medical University of South Carolina (Charleston, SC). Quantitation of the target GlcCer/GalCers, for which no synthetic standards are available, was accomplished using calibration curves of the closest counterpart.

Cholesterol and triglyceride levels were measured in kidney homogenates using the Cholesterol/Cholesterol Ester Quantification Kit (Biovision, San Francisco, CA) and Triglyceride Quantification Kit (Biovision), respectively, according to the manufacturer's instructions. Briefly, a two-phase extraction of 400 μg kidney homogenate was performed in 50 μl PBS; then, 200 μl chloroform/isopropanol:NP-40 (7:11:0.1 vol/vol) was added, vortexed for 2 minutes, and centrifuged. The organic phase was transferred to a new tube, dried down under nitrogen gas, and placed in a vacuum for 30 minutes to remove all remaining chloroform. The dried lipids were resuspended in 200 μl assay buffer and sonicated until homogenous (roughly 1 minute); 50 μl (100 μg worth of protein homogenate) was used for the assay on the basis of prior determination showing that this amount of protein had levels in the linear range of the kit. Levels were measured according to the manufacturer’s instructions.

**Proteinuria**

Urine was collected from mice over a 24-hour period in metabolic cages. To inhibit bacterial growth, ampicillin, gentamicin, and chloramphenicol were added to the collection tubes. Urinary protein levels were measured by albumin ELISA. Albumin was measured in 96-well plates coated overnight at 4°C with goat anti-mouse albumin. Wells were blocked with PBST/0.05% Tween-20 (PBST) plus 0.25% gelatin for 60 minutes at room temperature and washed with PBST; 2-fold dilution series of each urine sample were made in PBST/0.2% gelatin. For most samples 1:100–1:6400 was sufficient to obtain readings within the linear range of the standard curve, whereas some samples required additional dilution. Standards (mouse albumin; Sigma-Aldrich, St. Louis, MO) were serially diluted in a similar manner starting at 1 μg/ml. Standards and samples were incubated at room temperature for 60 minutes and washed with PBST. HRP-conjugated goat anti-mouse albumin (Fitzgerald Industries, Acton, MA) diluted 1:10,000 in PBST/0.25% gelatin was added to each well and incubated at room temperature for 60 minutes; then, the wells were washed with PBST. Substrate (100 μl/well 20 μl TMB/980 μl 0.2 M HCl, 100 ml 0.1 M citrate, pH 4.0, 50 μl H2O2) was added to each well and incubated at room temperature for 35 minutes. Absorbance was read at OD 380 nm on a Thermo Multiskan Ascent Plate Reader (Thermo Fisher Scientific), and albumin concentration per 1 ml urine was calculated from the standard curve using the Multiskan Ascent software. Three readings within the linear portion of the standard curve for each sample were averaged and multiplied by the total volume of urine to determine total albumin excreted in 24 hours.

**Gene Expression**

Reverse-transcribed RNA was used in real-time PCR to measure the relative message levels of GlcCer synthase, the major transcripts of LacCer synthases (GalT5, GalT6, and GalT7), glucocerebrosidase, GLA, ST3 β-galactoside α-2,3-sialyltransferase 5 (ST3GAL5 or GM3 synthase), NEU1 and NEU3, fatty acid synthase, 3-hydroxy-3-methylglutaryl-CoA reductase, and β-actin in RNA isolated from kidney cortices. Briefly, RNA was isolated from a 20-mg piece of kidney cortex from individual mice using the Qiagen RNeasy RNA Isolation Kit (Valencia, CA) according to the manufacturer’s instructions. RNA (1 μg) was reverse- transcribed using the Bio-Rad iScript cDNA Synthesis Kit (Hercules, CA) in a 20-μl reaction, and 1 μl cDNA was used per real-time PCR reaction. Each real-time reaction was performed in triplicate on the MyiQ Bio-Rad Thermocycler using the iQ SYBR Green Supermix (Bio-Rad), and threshold cycle number for each reaction was determined using the Real-Time iCycler iQ software (Bio-Rad). Relative expression was calculated using the Bio-Rad Gene Expression Macro software as described previously with β-actin as the reference gene. Relative differences between groups are presented as fold change. All primers were synthesized by IDT (Iowa City, IA). Primer efficiencies were determined for all primer sets to ensure similar efficiencies between each primer set for the gene.

### Table 1. Patient demographics and clinical measures of urine samples analyzed in Figure 7A and serum samples analyzed in Figure 7B

<table>
<thead>
<tr>
<th>Sample</th>
<th>Age (yr)</th>
<th>Women (%)</th>
<th>African American (%)</th>
<th>SLEDAI</th>
<th>eGFR</th>
<th>Activity</th>
<th>Chronicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine samples</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>37±11 (20–50)</td>
<td>86</td>
<td>71</td>
<td>4 (2–8)</td>
<td>116±42 (77–174)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SLE</td>
<td>37±16 (18–64)</td>
<td>100</td>
<td>67</td>
<td>10 (2–16)</td>
<td>73±40 (16–109)</td>
<td>6.5±6.4 (0–19)</td>
<td>2.6±1.7 (1–6)</td>
</tr>
<tr>
<td>SLE with nephritis</td>
<td>36±6 (29–46)</td>
<td>86</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum samples</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>37±14 (20–62)</td>
<td>90</td>
<td>90</td>
<td>5 (0–16)</td>
<td>110±36 (49–167)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SLE</td>
<td>36±11 (18–54)</td>
<td>89</td>
<td>89</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SLE with nephritis</td>
<td>32±9 (20–48)</td>
<td>90</td>
<td>90</td>
<td>16 (8–26)</td>
<td>65±38 (20–118)</td>
<td>8.9±5.3 (1.5–19)</td>
<td>4.1±2.2 (1–8)</td>
</tr>
</tbody>
</table>

Average age, SLE Disease Activity Index (SLEDAI), activity, and chronicity (±SD with ranges in parentheses) are provided for each group. All other subjects not in the African American column were Caucasian.
of interest and the primer set for the reference gene (β-actin), and all were >98%. Primer sequences are shown in Table 2.

NEU Activity
Kidney homogenates were prepared, and protein concentration was measured as described above. NEU enzyme activity was measured in 50 μg kidney homogenate using the Invitrogen AmplexRed NEU Assay Kit following the manufacturer’s instructions. The kit detects activity of all four NEU enzymes. NEU activity is measured in arbitrary units, and differences between groups are presented as fold change.

Protein Expression
SREBP and NEU1 protein levels were measured by immunoblot analysis. For SREBP detection, 20 μg kidney homogenate was separated on a 10% SDS-PAGE Tris-EDTA gel (Bio-Rad) and transferred to PVDF membranes. The membranes were incubated with either anti–SREBP-1 or anti–SREBP-2 mouse mAbs (SREBP-1 [2A4] and SREBP-2 [1C6]; Santa Cruz Biotechnology, Santa Cruz, CA), washed, and incubated with anti-mouse–HRP secondary antibody (Sigma-Aldrich). SREBP was detected with the Pierce ECL Kit (Thermo Fisher Scientific).

For NEU1 detection, each sample was normalized to the concentration of creatinine so that the equivalent of 50 μg creatinine was loaded per lane. Proteins were separated on a 10% SDS-PAGE TGX gel (Bio-Rad) and transferred to PVDF membranes. The membranes were incubated with either anti–NEU1 (H-300; Santa Cruz Biotechnology), washed, and incubated with an Alexa Fluor 647 goat anti-rabbit IgG antibody highly crossadsorbed secondary (Molecular Probes; Invitrogen, Eugene, OR). NEU1 was detected by scanning on the Odyssey Infrared Imaging System (LI-COR, Lincoln, NE). Replicate blots incubated with the secondary alone showed little to no background.

MALDI-MS Imaging of Kidney Sections
Direct profiling of glycosphingolipid expression in kidney tissue sections was accomplished by MALDI-IMS using a dual-source 7T Solarix FT-ICR MS instrument as previously described.40,41 Relative concentrations of GlcCer and LacCer species were assessed simultaneously in the same sections by MALDI-IMS in frozen kidney sections. Kidney tissues (10 μm) were prepared on ITO-coated glass slides, rinsed in water, and desiccated. Dihydroxybenzoic acid matrix (0.2 M in 50% methanol/0.01% TCA) was applied using an Image-Prep sprayer (Bruker Daltonics, Billerica, MA). Spectra were acquired across the entire tissue section using a Solarix 7T FTICR Mass Spectrometer (Bruker Daltonics) in positive ion mode in the mass range m/z=200–2000 with a SmartBeam II laser operating at 1000 Hz, a laser spot size of 25 μm, and a raster width of 50 μm. For each laser spot, 500 spectra were averaged, and all data were normalized using root means square. Images of glycosphingolipid species were generated using FlexImaging 4.0 software (Bruker Daltonics). For identification of individual glycosphingolipid species, continuous accumulation of selected ions/collision-induced dissociation fragmentation was done with reference standards spotted on an MALDI plate with DHB matrix or directly on tissue. Continuous accumulation of selected ions allows for trapping of a specific ion of interest within the quadrupole of the Solarix 7T FT-ICR Mass Spectrometer. After accumulation, the selected ion was fragmented using collision-induced dissociation. Structural assignments were made after detection of specific fragmentation patterns (e.g., the sphingoid base at m/z=264; loss of 162/180 for hexose), compared with an in-house structure database,41 and crossvalidated with the Lipid Maps database.

Immunohistochemistry
Human renal biopsies (frozen and embedded in OCT) were sectioned at 10 μm. Slides were thawed at room temperature and fixed with acetone at −80°C. Slides were washed with PBST plus 1% goat serum (Vectastain kit; Vector Laboratories, Burlingame, CA) and blocked with 5% goat serum at 4°C. Slides were washed with PBST/1% goat serum and incubated with a 1:50 dilution of rabbit anti-CD17 (LacCer; Biorybt, San Francisco, CA) in PBST/1% goat serum overnight at 4°C in a humidified chamber. After washing with PBST/1% goat serum, the slides were incubated with a 1:200 dilution of goat anti-rabbit IgG-FITC secondary (Southern Biotechnology Associates, Birmingham, AL) in PBST/1% goat serum for 60 minutes in a humidified chamber. Slides were washed with PBST/1% goat serum and incubated with a 1:50 dilution of rabbit anti-CD17 (LacCer; Biorybt, San Francisco, CA) in PBST/1% goat serum overnight at 4°C in a humidified chamber. After washing with PBST/1% goat serum, the slides were incubated with a 1:200 dilution of goat anti-rabbit IgG-FITC secondary (Southern Biotechnology Associates, Birmingham, AL) in PBST/1% goat serum for 60 minutes at room temperature in a humidified chamber. Sections were washed with PBST/1% goat serum, a drop of DAPI (Vectashield; Vector Laboratories) was added, and a coverslip was placed over sections followed by a final incubation for 15 minutes at room temperature in the dark. Images of sections were taken using a Nikon Eclipse 80i microscope, DS camera, and software (Nikon Instruments, Melville, NY).

<table>
<thead>
<tr>
<th>Table 2. Primer sequences for real-time PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Target Gene</strong></td>
</tr>
<tr>
<td>GlcCer Synthase (UGCG)</td>
</tr>
<tr>
<td>B-4-GalT-5</td>
</tr>
<tr>
<td>B-4-GalT-6</td>
</tr>
<tr>
<td>B-4-GalT-7</td>
</tr>
<tr>
<td>GM3 synthase (ST3 GalS)</td>
</tr>
<tr>
<td>Neu1</td>
</tr>
<tr>
<td>Neu3</td>
</tr>
<tr>
<td>GLA</td>
</tr>
<tr>
<td>Glucoscerbrosidase</td>
</tr>
<tr>
<td>Fatty acid synthase</td>
</tr>
<tr>
<td>3-Hydroxy-3-methyl-glutaryl-CoA reductase</td>
</tr>
<tr>
<td>β-Actin</td>
</tr>
</tbody>
</table>
Statistical Analyses
Statistical analyses were performed using GraphPad PRISM 6 software (GraphPad Software, Inc., La Jolla, CA). Nonparametric one-way or two-way ANOVA was performed with adjustment for multiple comparisons as appropriate. All analyses were unpaired, except for the proteinuria and LacCer longitudinal analyses in urine samples collected from the same mice over time presented in Figure 6. Specific statistical analyses used and $P$ values are indicated in the figures.

ACKNOWLEDGMENTS
The authors thank Dr. Darwin Bell and Stacy Steele for assistance with the confocal images and Dr. Paul Nietert for guidance with the statistical analyses.

Support for these studies was provided by National Institutes of Health National Institute of Arthritis & Musculoskeletal & Skin Diseases Grant P60-AR062755 Multidisciplinary Clinical Research Center Pilot Project (to T.K.N.) and Patient Resource Core (to J.C.O.), South Carolina Clinical and Translational Research (SCTR) Institute, with an academic home at the Medical University of South Carolina, through National Institutes of Health Grant Numbers UL1-RR029882 and UL1-TR000062, Veteran’s Administration Merit Review Grants BX000115 (to T.K.N.) and CX000218 (to J.C.O.), National Institutes of Health NIAMS Grant R01-AR45476-11 (to J.C.O.), the Alliance for Lupus Research (to J.C.O.), National Institutes of Health National Cancer Institute Grant R01-CA135087 (to R.R.D.), Department of Dermatology (to J.C.O.), Department of Defense Grant W81XWH-10-1-0136 (to R.R.D.), National Institutes of Health National Center for Research Resources Grant P20-RR17677 Center of Biomedical Research Excellence in Lipidomics and Pathobiology Pilot Project (to L.J.S.), National Institutes of Health National Institute of Diabetes and Digestive and Kidney Diseases Grant R01-DK093462 (to L.J.S.), and the Lipidomics Shared Resource of the Hollings Cancer Center at the Medical University of South Carolina supported by Cancer Center Support Grant P30CA138313.

The contents of this publication do not represent the views of the Department of Veterans Affairs, the US Government, or any of the other funding entities.

DISCLOSURES
None.

REFERENCES
24. Drappa J, Brot N, Elkon KB: The Fas protein is expressed at high levels on CD4+CD8+ thymocytes and activated mature lymphocytes in normal mice but not in the lupus-prone strain, MRL/lpr/lpr. Proc Natl Acad Sci U S A 90: 10340–10344, 1993


