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TITLE: Glycosphingolipids as therapeutic targets and biomarkers of lupus nephritis

PRINCIPAL INVESTIGATOR: Tamara Nowling, PhD

CONTRACTING ORGANIZATION: Medical University of South Carolina

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14. ABSTRACT The oseltamivir phosphate (OP) treatment of the MRL/lpr lupus prone strain has been completed for half of the mice and most samples analyzed, including proteinuria, renal pathology, circulating activated T cell numbers, kidney glycosphingolipid levels, and spleen weight and length. Treatment of the remaining half of the MRL/lpr mice was just completed and sample analysis will begin immediately. The B6.SLE1/2/3 genetic study (Neu1+/+ vs Neu1+/- mice) is underway and nearing completion. For the exosome biomarker study, exosomes have been isolated from most of the urine samples and proteomic and lipid discovery analyses run on a subset of samples. Proteomics analysis of this subset identified several proteins previously reported/validated as urine biomarkers of lupus nephritis. Additionally, several proteins were detected predominantly in treatment responders compared to non-responders or predominantly in non-responders compared to responders, which may be useful as biomarkers of response to therapy. These results are being confirmed in the remaining samples. In addition, we are investigating the role of NEU1 in the function of renal mesangial cells using cells isolated from the Neu1+/+ and Neu1+/- mice and have demonstrated that NEU mediates cytokine release in part through a TLR4-MAPK p38 pathway in cells stimulated with lupus serum.					
15. SUBJECT TERMS Lupus nephritis, Glycosphingolipid, Biomarkers, Neuraminidase					
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TABLE OF CONTENTS

	<u>Page</u>
1. Introduction	4
2. Keywords	4
3. Accomplishments	4
4. Impact	9
5. Changes/Problems	9
6. Products	10
7. Participants & Other Collaborating Organizations	11
8. Special Reporting Requirements	12
9. Appendices	13

1. INTRODUCTION:

In general, the initiating events of lupus are universally accepted to involve a genetic predisposition and an environmental trigger. Our data indicates that increased glycosphingolipid (GSL) levels in the kidney and urine of lupus mice and patients with nephritis is due to local renal increases in GSL metabolism following immune complex deposition. Altered GSL metabolism is unlikely to be a causative event in lupus nephritis, but GSLs likely play a pathogenic role in disease progression and are clear biomarker targets to monitor treatment efficacy and disease progression. Long-term goal and scope of the research includes targeting key molecules leading to reduction of renal damage to slow or prevent the progression of nephritis in lupus and other chronic kidney diseases, a major health concern in the Veteran population. The purpose (short-term goals) of this project is to 1) identify early urine biomarkers that will allow for earlier intervention to identify patients who fail therapy and/or prevent flares in lupus patients; and 2) demonstrate that the GSL catabolic pathway is a pathophysiological mechanism and novel target for therapeutic intervention in LN with relevance to other inflammatory kidney diseases. Our studies will have a profound impact on patients and the field of lupus by identifying specific mediators and biomarkers of renal pathology and opening up additional avenues of investigation in LN with applications to other renal diseases.

2. KEYWORDS:

Lupus nephritis, Glycosphingolipid, Biomarkers, Neuraminidase

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Major Task 1: Treatment of lupus mice with Oseltamivir. MRL/lpr mice were generated in our mouse colony, aged to 12-13 weeks, and treated with water or Oseltamivir Phosphate (OP), a neuraminidase inhibitor. The mice were treated in two sets to accommodate schedules and use of limited numbers of metabolic cages for collecting urine. In addition, samples (urine and blood) were collected from control mice (MRL/MpJ). Urine, blood, kidneys, and spleens were collected for all mice. The study has been completed including data analyses and a manuscript describing the study was published earlier this year.

Major Task 2: Genetic knockout of Neu1 in lupus mice. The B6 Neu1 knockout was backcrossed onto the B6.SLE1/2/3 strain, a lupus prone strain with a genetic knock out of one allele of the neuraminidase gene Neu1 (Neu1+/-). This study was a comparison of B6.SLE1/2/3 Neu1+/+, B6.SLE1/2/3 Neu1+/-, and healthy control C57BL/6 (B6) mice. All samples collected and sample analysis have been completed for this study. A manuscript (provided in the Appendix) has been drafted and submitted for review.

Major Task 3: GSLs as a predictor of response to therapy. Exosomes were isolated from urine samples of 157 lupus nephritis patients. Lipid analyses measuring hexosylceramide and lactosylceramide in all samples was completed. Proteomics on a subset of 10 samples was performed and proteins of interest identified, and a subset of those proteins further screened by individual ELISAs. Two proteins from the screen were chosen for analysis across all samples using individual ELISAs, which has been completed. The collected data is currently being analyzed by biostatistician Dr. Beth Wolf, a co-Investigator on this study, and a manuscript is being drafted.

Major Task 4: Association of renal and urine GSLs with renal function. Sectioning of biopsy samples have been completed for MALDI-FTICR analyses. Unfortunately, many of the needle biopsy samples we received either did not have enough tissue available or the tissue quality was too poor for MALDI-FTICR analyses.

What was accomplished under these goals?

The major activities completed during the no-cost extension award period included: 1) Acceptance of the manuscript describing the MRL/lpr OP treatment study in the journal *PLOS One* (provided in the Appendix); 2) completion of the genetic study, drafting of a manuscript, which is currently under review at the journal *Autoimmunity* (provided in the Appendix); 3) analyses of all LN patient urine exosome samples for two proteins and two glycosphingolipids was completed, and the data is currently being analyzed by a biostatistician as potential biomarkers of therapeutic response; and 4) sectioning of renal biopsies have been completed, but unfortunately, the samples either did not contain enough tissue or quality was too poor to be used for MALDI-FTICR. However, co-Investigator Dr. Drake who was to perform the MALDI-FTICR on the biopsies is instead running MALDI analyses on the urine exosome samples to examine changes in N-glycans between therapeutic responders and non-responders. Thus, we anticipate additional data that may be useful in the biomarker efforts. The specific objectives for this past year were to 1) complete the genetic mouse study in the B6.SLE1/2/3 (Neu1^{+/+} vs Neu1^{+/-}) lupus strain and draft a manuscript, 2) publish a manuscript describing the MRL/lpr treatment study, 3) complete the analyses of the human urine exosomes in the biomarker of therapeutic response study, 4) complete the MALDI-FTICR analyses of the renal biopsies. All tasks were completed except for the MALDI-FTICR analyses of the renal biopsies. In addition to the Major Task, a side study analyzing the role of neuraminidase (NEU), an enzyme that regulates glycosphingolipid metabolism, was examined in primary mouse mesangial cells. Those studies resulted in a manuscript recently accepted for publication in the journal *Immunology* (provided in the Appendix), and additional data included in manuscript describing the B6.SLE1/2/3 genetic study currently under review.

Accomplishments and findings for the entire award period for each Major Task include:

Major Task 1 (completed): Results are presented in a paper published in *PLOS One* (provided in the Appendix).

A summary of the findings include:

- 1) MRL/lpr mice (water treated) with nephritis had significantly elevated renal levels of ganglioside GM3 compared to age-matched MRL/MpJ mice (lupus mice without nephritis). GM3 had not been measured previously and the elevated levels were unexpected since we previously demonstrated activity of the enzyme that catabolizes GM3 (neuraminidase 1 or NEU1) is elevated in the kidney of nephritic MRL/lpr mice.
- 2) MRL/lpr mice (water treated) with nephritis had significantly elevated renal levels of sphingolipids that feed into the glycosphingolipid (GSL) pathway through Ceramide; Sphingosine 1 phosphate (S1P), Sphingosine (So), Dihydrosphingosine 1 phosphate (DHS1P), and Dihydrosphingosine (DHSO), compared to age-matched MRL/MpJ mice (lupus mice without nephritis).
- 3) MRL/lpr lupus mice treated with an inhibitor of NEU activity (OP) had slightly increased levels of some chain lengths of the glycosphingolipids hexosylceramides (HexCers), lactosylceramides (LacCers), and ganglioside GM3s in the kidney compared to MRL/lpr mice treated with water. We expected HexCers and LacCers would be reduced due to decreased activity of NEU in catabolizing GM3 to LacCers (and LacCers to HexCers).
- 4) There was no effect on disease measures in the MRL/lpr mice treated with OP compared to mice treated with water. This included proteinuria (urine albumin levels), renal pathology, splenomegaly, serum IgG, activated T cell numbers.
- 5) This manuscript also includes a pilot treatment study (supported by an internal pilot award) performed several years ago treating MRL/lpr mice with an inhibitor of GlcCer synthase to block GSL synthesis. Original analysis of samples from this study appeared to indicate no effect of the inhibitor on renal disease measures. However, we further analyzed the renal samples from these mice and observed a significant dose-dependent effect in reducing levels of all or most chain

lengths of HexCers and ganglioside GM3s and a trend of reduced LacCers levels. Importantly, analysis of serum and spleens from these mice that were stored showed a dose-dependent decrease in splenomegaly and trend of decreased serum IgG levels.

- 6) We concluded that blocking the GSL catabolism pathway by inhibiting NEU activity failed to reduce renal GSL levels (and actually further increased levels) and did not improve disease, while blocking GSL synthesis reduced renal GSL levels and showed significant or trends of improved disease measures in the MRL/lpr lupus mouse strain.
- 7) We previously reported that NEU activity could mediate the secretion of IL-6 in MRL/lpr lupus prone MCs when stimulated with a mimic of immune complexes. We also then continued these studies over the past year to garner a better understanding the mechanisms by which NEU activity mediates IL-6 secretion from MCs. Results from these experiments have been included in a manuscript recently accepted for publication in the journal *Immunology* (accepted manuscript included in the appendix). In summary, we demonstrated that in addition to an immune complex mimic, lupus serum stimulates secretion of IL-6 (and also GM-CSF and MIP1 α) from MRL/lpr lupus prone MCs and this secretion is mediated by NEU activity. We further showed that NEU activity mediates IL-6 expression at the message level and that NEU-mediated IL-6 secretion occurs in part through TLR4 and the p38/ERK MAPK signaling pathway in response to lupus serum and TLR4 ligand LPS.

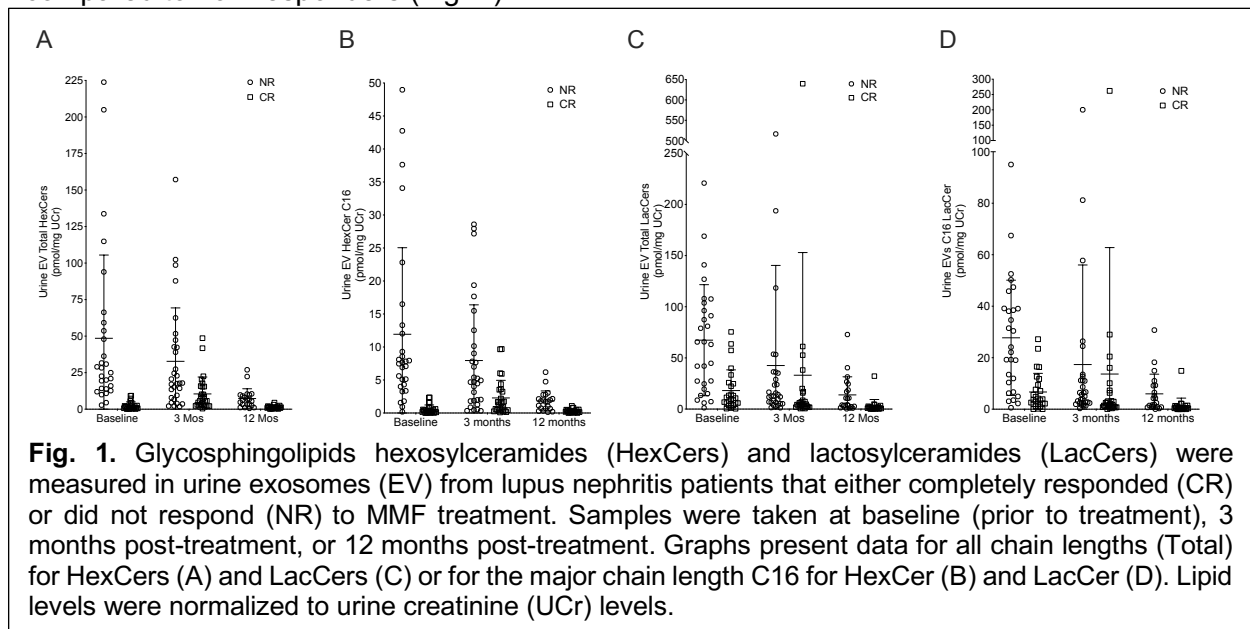
Major Task 2 (completed): Results are presented in drafted manuscript (provided in Appendix) submitted to the journal *Autoimmunity*. A summary of the major findings include:

- 1) Knocking out one allele of the GSL catabolic enzyme NEU1 (*Neu1*^{+/-}) in B6.SLE1/2/3 lupus prone mice did not significantly impact overall renal NEU activity, suggesting activity of NEU1 itself was increased, or the other major renal NEU (NEU3) expression level and/or activity was increased, to compensate for the reduced expression levels of NEU1. Renal levels of HexCers and LacCers also were not significantly altered in the *Neu1*^{+/-} mice. However, the lupus prone B6.SLE1/2/3 mice developed only mild renal disease by 44 weeks of age, an age at which most mice in this lupus strain have succumbed to disease (see point #2 below).
- 2) Knocking out one allele of the GSL catabolic enzyme NEU1 (*Neu1*^{+/-}) in B6.SLE1/2/3 lupus prone mice did not significantly impact proteinuria (urine albumin), renal pathology, and *Il-6* expression as these measures were similar between the *Neu1*^{+/+} and *Neu1*^{+/-} B6.SLE1/2/3 mice. However, as stated in point #1, most of these mice developed only very mild renal disease. Most B6.SLE1/2/3 lupus prone mice had levels of proteinuria only slightly higher than the non-lupus prone C57BL/6 healthy control mice. Renal pathology scoring is pending.
- 3) Measures of systemic disease, including splenomegaly and serum IgG at the study endpoint (44 weeks of age), were significantly higher in the B6.SLE1/2/3 mice compared to the C57BL/6 healthy controls. However, these measures were not significantly different in the B6.SLE1/2/3 *Neu1*^{+/+} compared to the *Neu1*^{+/-} mice. Activated T cell numbers, another hallmark of disease also were not different between the *Neu1*^{+/+} and *Neu1*^{+/-} mice.
- 4) We conclude reducing *Neu1* expression by knocking out one allele of *Neu1* in the B6.SLE1/2/3 lupus prone strain reduces NEU1 protein levels, but did not appear to significantly decrease NEU activity in the kidney. Importantly, knocking out one allele of *Neu1* does not alter the levels of the renal GSLs HexCers and LacCers, measures of renal disease, or measures of systemic disease. The B6.SLE1/2/3 mice typically develop high levels of proteinuria with 50% of mice dead due to nephritis by 36 weeks of age. However, disease was extremely delayed in this strain in our facility with low levels of proteinuria in a majority of the mice at 44 weeks of age and only mild increases in systemic disease measures. Thus, it's possible an impact of reducing *Neu1* expression on disease measures may be observed in more advanced disease.

- 5) Additional analyses of the B6.SLE1/2/3 Neu1^{+/+}, B6.SLE1/2/3 Neu1^{+/-}, and B6 mice examining sialylated glycans in renal tissue section identified interesting differences between the lupus B6.SLE1/2/3 mice and healthy B6 mice. The B6.SLE1/2/3 mice had decreased levels of N-glycans with α 2,6 linked sialic acids as assessed by lectin binding and this decrease was partially rescued by genetically reducing Neu1 expression in the B6.SLE1/2/3 Neu1^{+/-} mice. This data was included in the manuscript.
- 6) Results from this genetic study are included in a drafted manuscript that also includes results from experiments performed *in vitro* on primary mesangial cells (MCs) isolated from C57BL/6 Neu1^{+/+} and C57BL/6 Neu1^{+/-} mice. The *in vitro* results demonstrate that knocking out one allele of Neu1 significantly reduces NEU activity in MCs and impacts their function. Specifically, Neu1^{+/-} MCs released significantly less IL-6 and GM-CSF and expressed significantly lower levels of *Il-6* and *Gm-csf* message levels in response to lupus serum or LPS stimulation.

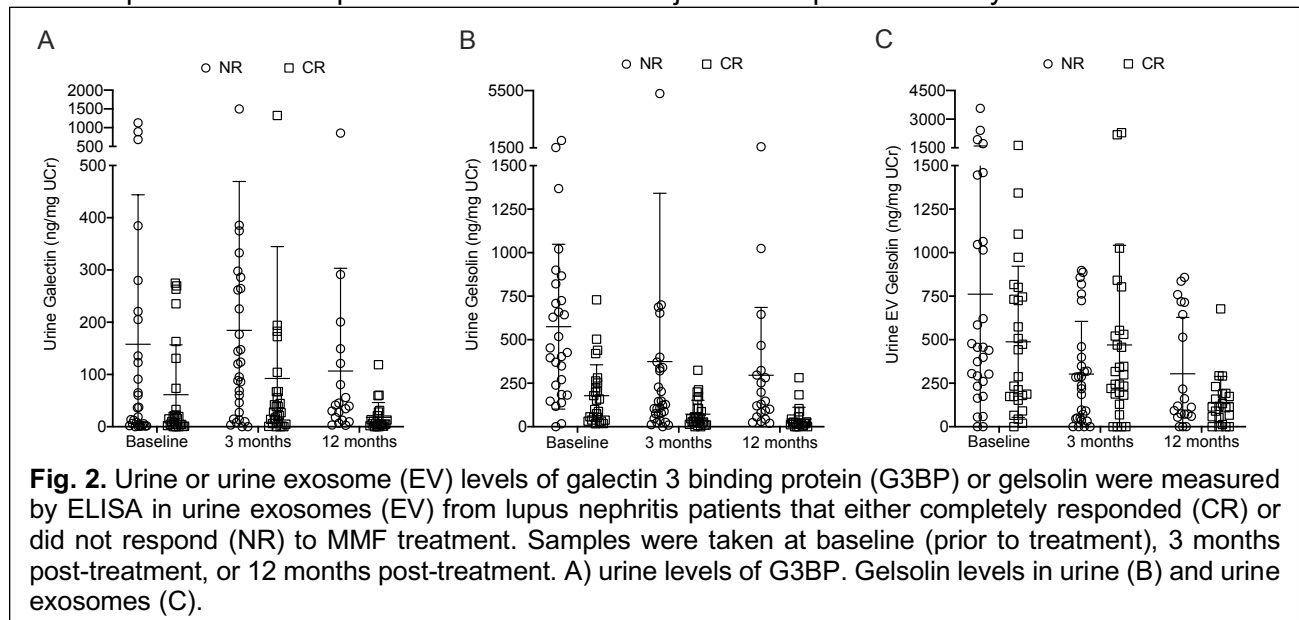
Major Task 3 (nearly complete): Major findings from this study includes:

- 1) Exosomes were isolated from longitudinal urine samples of ~60 lupus nephritis patients (30 complete responders and 30 non-responders) treated with mycophenolate mofetil. Samples were collected: 1) prior to treatment, 2) 3 months after treatment, and 3) 12 months after treatment. Patients were divided into complete responders and non-responders to treatment as assessed at 12 months post-treatment.
- 2) Glycosphingolipids HexCers and LacCers were measured in all exosome samples demonstrating that responders had significantly lower levels of HexCers at baseline (prior to treatment) compared to non-responders (Fig. 1).



- 3) Four novel proteins identified as being either elevated or reduced in the complete responders compared to the non-responders in a proteomics analysis performed on a subset of 10 samples, and one previously identified biomarker of LN, were screened with individual ELISAs. Two proteins: 1) galectin 3 binding protein (G3BP), previously identified biomarker for LN, in urine and 2) gelsolin, not previously measured in LN, in urine or urine exosomes were readily detected by the individual ELISAs and subsequently measured in all samples. G3BP levels in urine are similar at all time points between responders and non-responders indicating this protein is likely a biomarker of disease and not useful for differentiating responders/non-responders (Fig. 2A).

Gelsolin in complete responders is lower in urine at all time points (Fig. 2B), but similar in urine exosomes (Fig. 2C) compared to non-responders. Statistical analyses of all the biomarker data is nearing completion and a manuscript is currently being drafted. A completed manuscript is anticipated to be completed and submitted to a journal for peer review by 1/30/2021.



Major Task 4 (not completed): Due to inadequate amounts of tissue or poor tissue quality, most of the renal biopsies obtained for this study did not produce data in the MALDI-FTICR analyses conducted by co-Investigator Dr. Drake. However, there was enough sample remaining from most of the urine exosome samples that further analyses are currently being performed. Based on observations in renal tissues from the B6.SLE1/2/3 genetic study that indicated N-glycans are altered in the kidney of lupus mice compared to healthy controls (see Major Task 2 results), we are examining N-glycan levels in urine exosomes with respect to therapeutic response. This analysis is just beginning and is anticipated to be completed by the middle of 2021.

What opportunities for training and professional development has the project provided?

Over the course of the award period, the PI attended several scientific conferences to present findings from the DoD studies. This included the American Society of Nephrology Kidney Week in 2017 and 2018, the Southeastern Regional Lipid Conference in 2016 and 2017, and the American College of Rheumatology Annual Meeting in 2016, 2018 and 2019. Post-doctoral fellow Kamala Sundararaj and Research Specialist Jessalyn Rodgers, supported by this award, also presented findings from this study at several regional or local conferences including the Southeastern Regional Lipid Conference, MUSC Translational Research Day, and MUSC Department of Medicine Research Day between 2017 and 2020.

How were the results disseminated to communities of interest?

In addition to the oral and poster presentations described above, results were also presented two to three times a year to several collaborative research groups to which the PI belongs and meets with on a regular basis on our campus. This includes a “Lupus Research Group”, an “Exosome Research Interest Group”, Rheumatology division. The PI also presents the research to the Rheumatology fellows as a means of educating the fellows concerning ongoing research questions and approaches used to address and translate the research to patient care.

What do you plan to do during the next reporting period to accomplish the goals?

N/A

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

Overall, the results from the entire award period demonstrate that inhibiting or reducing the enzyme neuraminidase, which regulates the breakdown pathway of glycosphingolipids, is not likely to be effective in reducing glycosphingolipids in the kidney or improve the development or progression of lupus. However, blocking the enzyme glucosylceramide synthase, which regulates the synthesis pathway of glycosphingolipids, shows promise as a potential therapeutic approach to reducing glycosphingolipid levels in the kidney and improving disease. On a cellular level, neuraminidase plays an important role in mediating the production of inflammatory mediators (cytokines) by mesangial cells in the kidney. We also identified in part the mechanism by which neuraminidase mediates this production. The significance of these observations is still under investigation since blocking neuraminidase does not appear to have a significant impact in the context of kidney disease in lupus mice. This knowledge opens up additional questions and avenues of investigation and suggests a large scale pre-clinical mouse study to fully test blocking glucosylceramide synthase in treating lupus is warranted. Importantly, our results analyzing patients with lupus nephritis suggest that elevated glycosphingolipids present in small vesicles called exosomes present in the urine may serve as a biomarker for identifying patients that are not likely to respond to treatment with mycophenolate mofetil before they even begin treatment. Further testing is planned to determine if these lipids may also identify patients not likely to respond to other treatment regimens, and whether “non-responding” patients may benefit from therapy with a glucosylceramide synthase inhibitor is used to reduce the glycosphingolipid levels in the kidney in combination with mycophenolate mofetil (or other therapies).

What was the impact on other disciplines?

The impact on other disciplines are speculative. However, we anticipate that our results will be relevant to other kidney diseases that are characterized by elevated or dysregulated glycosphingolipid metabolism.

What was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

Nothing to report

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to report.

Actual or anticipated problems or delays and actions or plans to resolve them

As stated above, the MALDI-FTICR analyses of the human kidney biopsies were unable to be performed due to the poor quality or lack of sufficient tissue (Major task 4).

Changes that had a significant impact on expenditures

Nothing to report

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Significant changes in use or care of human subjects

Nothing to report.

Significant changes in use or care of vertebrate animals

Nothing to report.

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Journal publications.

Published or accepted peer-reviewed articles (entire award period):

1. Sundararaj K, JI Rodgers, M Subathra, LJ Siskind, E Bruner, **TK Nowling**. 2018. Neuraminidase activity mediates IL-6 production by activated lupus prone mesangial cells. *American Journal of Physiology: Renal Physiology*, 314:F630-F642 (PMC5966761). Federal support acknowledged
2. **Nowling TK**, JI Rodgers, T Thiyagarajan, B Wolf, E Bruner, K Sundararaj, I Molano, G Gilkeson. 2020. Targeting glycosphingolipid metabolism as a potential therapeutic approach for treating disease in female MRL/lpr lupus mice. *PLoS ONE* 15(3):e0230499 (PMID: 32187230). Federal support acknowledged
3. Sundararaj K, JI Rodgers, P Angel, B Wolf, **TK Nowling**. The role of neuraminidase in TLR4-MAPK signaling and the release of cytokines by lupus serum-stimulated mesangial cells. *Immunology, manuscript accepted 12/7/2020* (DOI:10.1111/imm.13294; PMID: 33314123) Federal support acknowledged

Manuscripts submitted for peer review:

4. Rodgers JI, K Sundararaj, E Bruner, B Wolf, **TK Nowling**. The role of neuraminidase 1 (NEU1) in the function of primary mouse mesangial cells and disease outcomes in murine lupus nephritis. Submitted and under review, *Autoimmunity* Federal support acknowledged

Manuscripts in preparation:

5. Nowling TK, JI Rodgers, B Wolf, B Troyer, J Oates. Urine exosome factors as biomarkers of response to therapy in lupus nephritis.

Books or other non-periodical, one-time publications.

Nothing to report.

Other publications, conference papers and presentations.

Presentations for entire award period:

1. **Tamara Nowling**, Kamala Sundararaj, Jessalyn Rodgers, Leah Siskind, Richard Drake. 2016. NEU1-mediated glycosphingolipid catabolism regulates the function of mesangial cells. Southeastern Regional Lipid Conference, Cashiers, NC.

2. **Tamara Nowling**, Kamala Sundararaj and Leah Siskind. 2016. Immune Complex-Induced IL-6 Production by Lupus Prone Mesangial Cells is Mediated by Neuraminidase Activity. American College of Rheumatology Annual Meeting, Washington, D.C.
3. **Tamara Nowling**, Kamala Sundararaj and Jessalyn Rodgers. 2017. Neuraminidase Activity Mediates IL-6 Production by Activated Lupus Prone Mesangial Cells. American Society of Nephrology Kidney Week, New Orleans, LA.
4. Kamala Sundararaj, Jessalyn Rodgers, **Tamara Nowling**. 2017. NEU1-Mediated IL-6 Production by Mesangial Cells. Southeastern Regional Lipid Conference, Cashiers, NC.
5. Jessalyn Rodgers, Kamala Sundararaj, Richard Drake, Michael Janech, James Oates, **Tamara Nowling**. 2017. Glycosphingolipids as Biomarkers of Lupus Nephritis. Southeastern Regional Lipid Conference, Cashiers, NC.
6. **Tamara Nowling**, Jessalyn Rodgers, Jim Oates, Michael Janech, Richard Drake. 2018. Glycosphingolipid and Proteins in Urine Exosomes: Potential Biomarkers of Lupus Nephritis. American College of Rheumatology Annual Meeting, Chicago, IL.
7. **Tamara Nowling**, Kamala Sundararaj, Jessalyn Rodgers. 2018. Neuraminidase Activity Mediates IL-6 Secretion from Activated Lupus Mesangial Cells Through TLR4 and MAPK p38. American Society of Nephrology Kidney Week, San Diego, CA.
8. Jessalyn Rodgers, Kamala Sundararaj, **Tamara Nowling**. 2018. Glycosphingolipids as Biomarkers of Lupus Nephritis. Translational Research Day, Medical University of South Carolina.
9. Kamala Sundararaj, Jessalyn Rodgers, **Tamara Nowling**. 2019. NEU1 mediates IL-6 release by mesangial cells through TLR4-MAPK pathway. Department of Medicine Research Day, Medical University of South Carolina.
10. Jessalyn Rodgers, Evelyn Bruner, Bethany Wolf, **Tamara Nowling**. 2019. Targeting glycosphingolipid metabolism as a treatment for lupus. Department of Medicine Research Day, Medical University of South Carolina

- **Website(s) or other Internet site(s)**
Nothing to report.
- **Technologies or techniques**
Nothing to report.
- **Inventions, patent applications, and/or licenses**
Nothing to report.
- **Other Products**
Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

(Over the course of the entire award)

Name: Tamara Nowling, PhD

Project Role: PI

Research Identifier: ORCID ID: 0000-0002-1445-5415

Nearest person month worked: 7.2

Contribution to Project: Dr. Nowling directed planning of all experiments and analyses, met with and coordinated patient urine exosome sample analyses with co-investigators, and assisted with and/or

provided guidance with breeding, urine/blood/organ collections from study mice, exosome isolations, and sample analyses.

Name: Jessalyn Rodgers, MS

Project Role: Research Specialist

Nearest person month worked: 12

Contribution to Project: Ms. Rodgers' main duties have involved maintaining the breeding and genotyping of mice, collecting and analyzing samples from study mice, and exosome isolations/analyses. She has also been involved with an extension of the proposed studies in analyzing mechanisms by which NEU activity impacts primary mesangial cells isolated from the Neu1 knockout mice.

Name: Kamala Sundararaj, PhD

Project Role: Staff Scientist

Nearest person month worked: 12

Contribution to Project: Dr. Sundararaj assisted with exosome analyses, analyses of samples from the mouse studies, and performed the majority of the *in vitro* mechanistic studies of MRL/lpr and C57BL/6 mesangial cells.

Name: Rick Drake, PhD

Project Role: Co-investigator

Nearest person month worked: 0.6

Contribution to Project: Dr. Drake performed MALDI-FTICR analyses and assisted with exosome sample analyses.

Name: Beth Wolf, PhD

Project Role: Co-investigator

Nearest person month worked: 0.6

Contribution to Project: Dr. Wolf performed all the statistical analyses of the data from the mouse studies, most of the *in vitro* mesangial cell analyses, and is performing the statistical analyses of the urine exosome biomarker study.

Name: Jim Oates, MD

Project Role: Co-investigator

Nearest person month worked: 0.24

Contribution to Project: Dr. Oates provided guidance with the human urine exosome sample analyses and assisted with data analysis.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Tamara K. Nowling, PhD: No changes.

N/A for remaining senior/key personnel as the award was in a no-cost extension and no salary was provided for those individuals.

What other organizations were involved as partners?

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: N/A

QUAD CHARTS: N/A

9. APPENDICES:

- Sundararaj K, JI Rodgers, M Subathra, LJ Siskind, E Bruner, **TK Nowling**. 2018. Neuraminidase activity mediates IL-6 production by activated lupus prone mesangial cells. *American Journal of Physiology: Renal Physiology*, 314:F630-F642 (PMC5966761).
- **Nowling TK**, JI Rodgers, T Thiyagarajan, B Wolf, E Bruner, K Sundararaj, I Molano, G Gilkeson. 2020. Targeting glycosphingolipid metabolism as a potential therapeutic approach for treating disease in female MRL/lpr lupus mice. *PLoS ONE* 15(3):e0230499 (PMC7080257).
- Sundararaj K, JI Rodgers, P Angel, B Wolf, **TK Nowling**. The role of neuraminidase in TLR4-MAPK signaling and the release of cytokines by lupus serum-stimulated mesangial cells. *Immunology*, *manuscript accepted 12/7/2020* (DOI:10.1111/imm.13294; PMID: 33314123).
- Rodgers JI, K Sundararaj, E Bruner, B Wolf, **TK Nowling**. The role of neuraminidase 1 (NEU1) in the function of primary mouse mesangial cells and disease outcomes in murine lupus nephritis. Under review, *Autoimmunity*

RESEARCH ARTICLE

Neuraminidase activity mediates IL-6 production by activated lupus-prone mesangial cells

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Sundararaj K, Rodgers JI, Marimuthu S, Siskind LJ, Bruner E, Nowling TK. Neuraminidase activity mediates IL-6 production by activated lupus-prone mesangial cells. *Am J Physiol Renal Physiol* 314: F630–F642, 2018. First published December 20, 2017; doi: 10.1152/ajprenal.00421.2017.—The development of nephritis is a leading cause of morbidity and mortality in lupus patients. Although the general pathophysiological progression of lupus nephritis is known, the molecular mediators and mechanisms are incompletely understood. Previously, we demonstrated that the glycosphingolipid (GSL) catabolic pathway is elevated in the kidneys of MRL/lpr lupus mice and human lupus patients with nephritis. Specifically, the activity of neuraminidase (NEU) and expression of *Neu1*, an enzyme in the GSL catabolic pathway is significantly increased. To better understand the role and mechanisms by which this pathway contributes to the progression of LN, we analyzed the expression and effects of NEU activity on the function of MRL/lpr lupus-prone mesangial cells (MCs). We demonstrate that NEU1 and NEU3 promote IL-6 production in MES13 MCs. *Neu1* expression, NEU activity, and IL-6 production are significantly increased in stimulated primary MRL/lpr lupus-prone MCs, and blocking NEU activity inhibits IL-6 production. NEU1 and NEU3 expression overlaps IgG deposits in MCs in vitro and in renal sections from nephritic MRL/lpr mice. Together, our results suggest that NEU activity mediates IL-6 production in lupus-prone MCs possibly through an IgG-receptor complex signaling pathway.

IL-6; lupus nephritis; mesangial cells; neuraminidase

INTRODUCTION

Lupus is a systemic autoimmune disorder that arises with immune system malfunction affecting multiple organs and tissues. Disease is characterized by autoantibody production and immune complex deposition in target organs, leading to inflammation and organ damage. Though lupus affects skin, joints, heart, lungs, and brain, 30–60% of patients develop glomerulonephritis, a leading cause of morbidity and mortality. One of the earliest hallmarks of lupus nephritis (LN) is the deposition of immune complexes in the glomeruli, which leads to production of proinflammatory cytokines and chemokines by resident renal cells, immune cell infiltration, inflammation,

and eventually tissue damage (reviewed in Refs. 32 and 55). Despite understanding this general sequence of events that leads to renal damage in LN, the specific molecular mechanisms involved have not been completely elucidated, especially with respect to resident renal cell responses that contribute to the progression of disease. Identifying molecular mechanisms that play a role in renal inflammation and damage will support development of more effective therapeutic approaches for treating lupus patients with nephritis.

We previously reported that glycosphingolipid (GSL) metabolism is altered in the kidney and/or urine of lupus mice and humans with nephritis compared with their disease counterparts without nephritis and to healthy controls (34). Specifically, lactosylceramide (LacCer), glucosylceramide (GlcCer), neuraminidase (NEU) activity, and *Neu1* mRNA levels are increased. These results suggest that the catabolic pathway mediated by NEU activity is elevated in LN. NEUs, also known as sialidases, are enzymes distributed widely in different organisms from fungi to mammals that remove sialic acids from glycolipids and glycoproteins impacting cell signaling and function. By removing sialic acid residues from gangliosides (a class of sphingolipids), NEU activity can lead to upregulation of lactosylceramide and production of cytokines and chemokines, resulting in immune cell infiltration, inflammation, and tissue damage. In mammals, there are four NEU family members: NEU1, NEU2, NEU3, and NEU4. These enzymes differ in their tissue expression and cellular localization. NEU1 is expressed globally with highest expression observed in the pancreas and kidney, and it is located in the lysosome and the plasma membrane within the cell (7, 23, 25, 33). NEU3 is expressed most highly in skeletal muscle, testis, adrenal gland, thymus, and prostate, with lower levels observed in several other organs, including kidney, and is mainly associated with the plasma membrane (29). *Neu1* and *Neu3* mRNAs are more highly expressed in the kidney than the other *Neus* with *Neu1* expressed ~40-fold higher than *Neu3* and 80-fold higher than *Neu4* (52).

Here, we provide additional evidence that renal NEU activity is elevated and that NEU1 and NEU3 are highly expressed in the expanding mesangial cells (MCs) in renal sections from nephritic lupus mice. Therefore, we examined the role of NEU activity and potential mechanisms by which NEUs mediate the activation of lupus-prone MCs. We demonstrate that activation

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of lupus-prone primary MCs using aggregated IgG, a mimic of immune complexes, increases *Neu1* message levels, NEU activity, and IL-6 and MCP-1 production. Importantly, we show that IL-6 production by lupus-prone MCs activated by aggregated IgG or lupus serum is mediated by NEU activity. Further, we demonstrate overlapping expression of NEU1 and NEU3 with aggregated IgG binding at the surface of cultured MCs and with IgG deposits in renal sections of nephritic mice. Together, our results suggest that NEU activity may mediate (or modulate) IL-6 production in lupus MCs by interacting with an IgG-cell surface receptor complex. Targeting NEU activity may be a therapeutic approach to reduce renal inflammation in lupus.

MATERIALS AND METHODS

Mice. MRL/lpr and NZM2410 lupus mice between 8 and 34 wk of age (as indicated in the RESULTS) were used for all experiments. Both male and female NZM2410 and MRL/lpr mice were used for analyses in Fig. 1. The sex of mice used to generate primary MRL/lpr

mesangial cell lines is described below. C57BL/6 mice (14–16 wk of age) were used as a source of healthy serum. Mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and were maintained on a 12:12-h light-dark cycle with access to food and water ad libitum. All mice were housed under pathogen-free conditions at the animal facility of the Ralph H. Johnson Veterans Affairs Medical Center (Charleston, SC), and all animal experiments were approved by the Institutional Animal Care and Use Committee. Urine protein levels were determined using Chemstrip 7 (Roche Diagnostics, Indianapolis, IN).

Generation and culturing of primary mesangial cell lines. Kidneys were removed from three 6-wk-old (prenephritic) MRL/lpr mice and used for glomeruli isolation, as described previously (51) and cultured to grow out primary mesangial cells (MCs). Briefly, the medulla was removed, and the cortex was minced in PBS, incubated with collagenase, and passed sequentially through sieves of 180 μ m, 150 μ m, 75 μ m, and 40 μ m. The glomeruli on top of the 40- μ m sieve were collected and enriched from tubules by resuspending in media for 2 min, which allowed the glomeruli to sink to the bottom of the dish, while most of the tubules remained floating. The tubules were care-

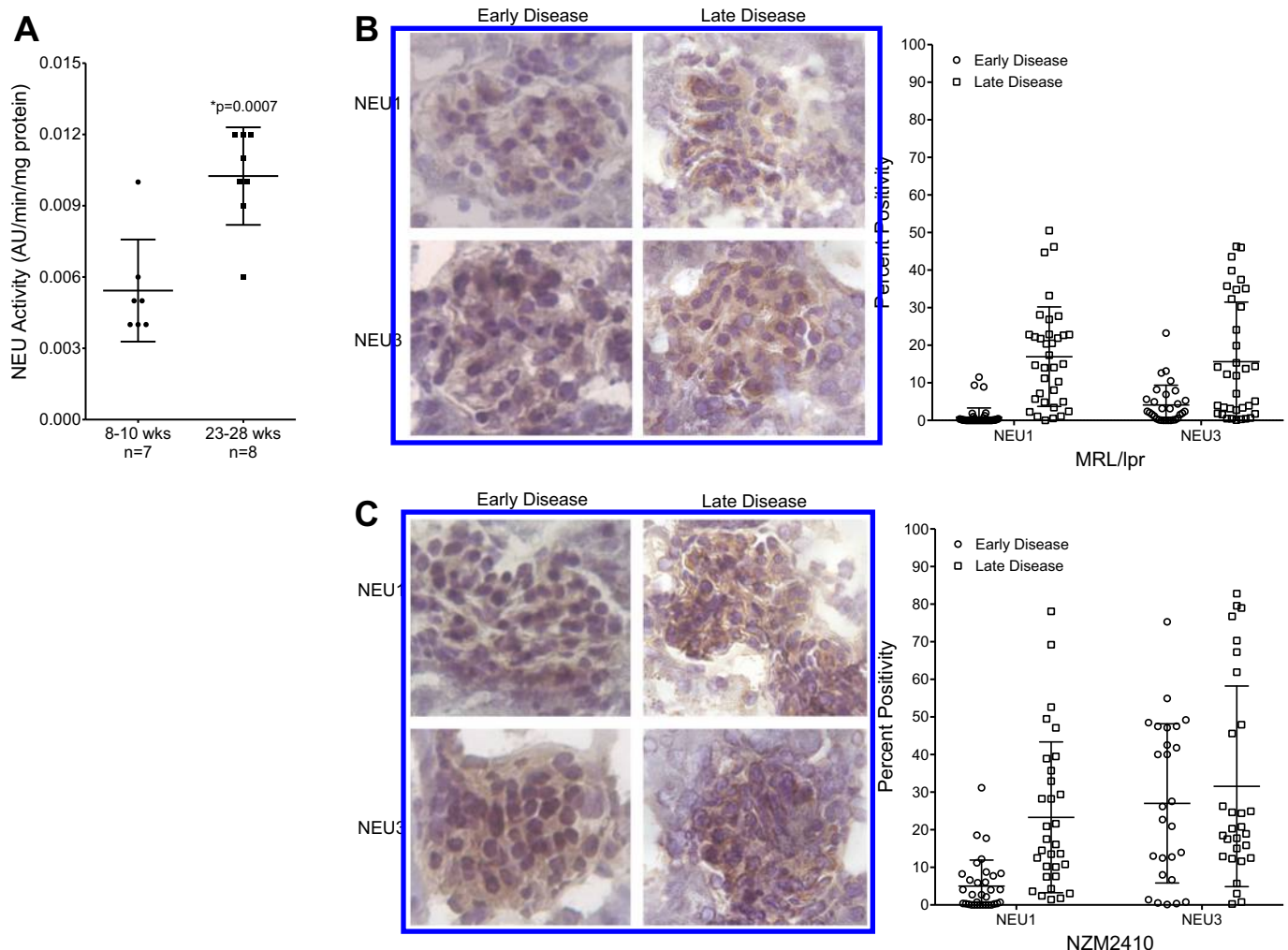


Fig. 1. Renal NEU activity is elevated and NEU1 and NEU3 are highly expressed in MCs of nephritic lupus mice. A: NEU activity was measured in kidney homogenates from 8- to 10-wk-old prenephritic and 23- to 28-wk-old nephritic NZM2410 mice. *P* value was calculated as described in MATERIALS AND METHODS. Immunohistochemistry analyses and semiquantitative measures of staining for NEU1 and NEU3 on renal sections of early and late disease stage MRL/lpr (B) and NZM2410 (C) mice. Images are representative of three mice from each group, which were evaluated by a pathologist. Percentage of high positive and positive NEU1- and NEU3-stained glomeruli are shown to the right of images. Staining in 8–12 glomeruli per animal were evaluated as described in MATERIALS AND METHODS with values of individual glomeruli presented.

fully removed with most of the media enriching the glomeruli to ~95% purity. The glomeruli were cultured at 37°C in a 5% CO₂ incubator in DMEM medium containing 10% FBS, 1% penicillin/streptomycin and ITS supplement (insulin 5 µg/ml, transferrin 5 µg/ml, and sodium selenite 5 ng/ml; Sigma-Aldrich, St. Louis, MO) for ~4 wk with medium changed every 4 or 5 days. After 3 wk, medium was supplemented with D-valine to inhibit fibroblast growth. Once stellate-shaped cells were observed, characteristic of MCs, they were trypsinized with trypsin/EDTA (Sigma-Aldrich) and grown in MEM/D-valine medium (GenDEPOT, Houston, TX).

Two independent MC lines were generated. One line was established from glomeruli isolated from two female and one male mice. The second line was generated from three female mice. Results from experiments stimulating each line and treating with the NEU inhibitor oseltamivir phosphate showed similar trends with data presented from the female line. Each primary MC line was characterized by flow cytometry at passage five, by IHC at passage six, and by RT-PCR at passages 5–9. Cell lines used were confirmed positive for MC markers (α SMA and PDGFR β), and negative for endothelial cell markers (CD144 and CD31) and podocyte cell markers (*nephrin* and *synaptopodin*). Lines used for experiments were 93% positive for α SMA by flow cytometry. RT-PCR results across passages 5–9 showed average relative expression levels of 16.5 for α SMA and 1.5 for PDGFR β . CD31 and CD144 did not show amplification until 32–33 cycles and were 0.04 and 0.06, respectively, relative to α SMA and PDGFR β . *Nephrin* was below the level of detection. All cell lines tested negative for mycoplasma. Cells at passages 6–9 were used in all experiments. MC lines were shown to express *Neu1* and *Neu3* with message levels of *Neu1* being 12–14-fold higher than *Neu3*.

Heat-aggregated IgG preparation and treatment of cells. Mouse IgG (Sigma-Aldrich) was heated at 63°C in a water bath for 30 min, placed directly on ice for 5 min, agitated mildly at 4°C overnight, and centrifuged to remove insoluble complexes. The final concentration of heat aggregated IgG (HA-IgG) was determined by NanoDrop spectrophotometry and aggregation confirmed by SDS-PAGE. HA-IgG preparations were tested for endotoxin using the Pierce Limulus Amebocyte Lysate Chromogenic Endotoxin kit (Thermo Fisher Scientific, Waltham, MA) and found to be <4.5 EU/ml. MCs were treated with increasing concentrations of HA-IgG or vehicle (sterile PBS) or with increasing amounts of serum from 16- to 18-wk-old (nephritic) MRL/lpr lupus mice or age-matched healthy C57BL/6 mice, as indicated in the figures. To ensure that the MRL/lpr serum was not contributing to the measured IL-6 and MCP-1 levels in the lupus serum studies, the highest percentage of MRL/lpr serum used (20%) was added to culture media without cells and compared with levels in cells cultured without MRL/lpr serum, which were not significantly different (data not shown). For the time course experiments, MCs were treated with 22.5 µg/ml HA-IgG for 1, 3, 6, 18, 24, and 48 h. For the neuraminidase inhibition experiments, oseltamivir phosphate (OP) (Santa Cruz Biotechnology, Santa Cruz, CA) was added to the culture medium at the concentrations indicated in the figures for 24 h before stimulation with 22.5 µg/ml HA-IgG or 20% MRL/lpr serum for 6 h. Media were collected at 3 h and 6 h for IL-6 and MCP-1 analyses. Although the extent of IL-6 and MCP-1 production varied among HA-IgG preparations, the dose-dependent trends were similar, and presented data are representative of at least three independent experiments per primary MC line. Trypan blue staining was used to ensure treatments did not adversely affect cell numbers. For the siRNA studies, cells were transfected with 200 nM siGENOME SMARTpool mouse *Neu1* siRNA or nontarget siRNA (Dharmacon, St. Louis, MO) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) 16 h before stimulation with heat-aggregated IgG or vehicle. Twenty-four hours after stimulation, media were collected and analyzed for cytokine production, cells were collected and used to measure *Neu1* and *Neu3* message levels, as described below.

Culturing, treatment, and transfection of MES13 mouse mesangial cell line. MES13 mouse mesangial cells (American Type Culture Collection, Manassas, VA) were cultured in low-glucose DMEM: Ham's F12 (3:1) medium supplemented with 5% FBS and 1% penicillin and streptomycin. For treatment of mesangial cells with glycosphingolipids, 0.5×10^6 cells were plated in 10 cm² dishes in complete growth media. Forty-eight hours after plating, the media were replaced with complete media containing 0.1 µM glycosphingolipids (an equal mixture of C₈-glucosylceramide and C₈-lactosylceramide, reconstituted in DMSO). After 1 h, the cells were washed with PBS and harvested by scraping in 700 µl of RLT buffer (Qiagen RNeasy mini kit, according to the manufacturer's instructions) for RNA isolation and real-time PCR, as described below. In all cases, an equal volume of DMSO was used as a vehicle control.

For transfections, cells were seeded in six-well plates one day before transfection. Transfections were performed using the Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions using pCMV *Neu1* or pCMV *Neu3* expression plasmids (Origene, Rockville, MD). The pCMV empty construct was used alone and added to all transfection experiments when necessary to ensure equimolar amounts of total DNA per well. Plasmids were isolated from bacterial stocks using an endotoxin-free plasmid isolation kit (Qiagen, Frederick, MD) following manufacturer's instructions. Media were collected 24 h and 48 h after transfection for measuring IL-6 and MCP-1. Cells were harvested 48 h after transfection, and total protein was measured for normalizing IL-6/MCP-1 levels. Trends were the same at 24-h and 48-h time points; data from the 48-h time point are presented. For the HA-IgG experiments, HA-IgG was added in increasing amounts 6 h after transfection with 4 µg of the indicated plasmid, and the cells were cultured for an additional 24 h. Trypan blue staining was used to ensure transfections and HA-IgG treatments did not adversely affect cell numbers. Cultures tested negative for mycoplasma.

ELISA. IL-6 and MCP-1 were quantified in medium of cultured cells using ELISA kits, according to the protocol provided by the manufacturer (Biolegend, San Diego, CA) with minimum detectable concentrations of 2 pg/ml for IL-6 and 30 pg/ml for MCP-1. Treatments of the primary MCs did not appear to cause excessive cell death or alter cellular phenotype as assessed by Trypan blue staining. However, MES13 cells transfected with 8 µg of pCMV *Neu1* or pCMV *Neu3* appeared to have altered phenotypes, and the total protein content was slightly lower compared with transfections using lower amounts of the *NEU1* and *NEU3* expression plasmids. Concentrations of IL-6 and MCP-1 for all experiments, except the siRNA experiments, were normalized to total cellular protein content to account for any effects of treatments with final concentrations presented adjusted to an equivalent of 25 µg of protein after normalization for comparison across experiments. For the siRNA experiments, cytokine levels are presented per 75,000 cells.

Semiquantitative real-time RT-PCR assays. RNA was isolated from MCs using the RNeasy kit (Qiagen, Hilden, Germany) following the manufacturer's directions, and then cDNA was reverse transcribed with 0.5–1 µg RNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). For the cytokine/chemokine gene analyses, real-time PCR was performed using Bio-Rad iQ SYBR Green Super mix, and the Bio-Rad CFX 96 real-time system (C1000 Touch Thermal cycler). The fluorescence was measured during steps 2 and 4. All reactions were performed in triplicate and normalized to GAPDH. For the *Neu1*, *Neu3*, and MC marker gene analyses, real-time PCR was performed using the LightCycler 480 SYBR Green I Master kit and LightCycler 480 II (Roche). All reactions were performed in triplicate and normalized to β actin. No amplification was observed in the negative control PCR reactions using nonreverse transcribed RNA. Relative message levels were calculated using the $\Delta\Delta$ CT method and are presented as the means of normalized expression. The mean of normalized expression is directly proportional to the amount of RNA of the target gene relative to the amount of RNA of the reference gene

or internal control (*βactin* or *GAPDH*). The $\Delta\Delta CT$ for *Neu1* for all conditions were compared with the vehicle-treated condition for *Neu1* (0 $\mu\text{g/ml}$ HA-IgG), which was set to one. *Neu3* message levels are presented relative to *Neu1* message level in the vehicle-treated condition. All real-time PCR data presented are an average of three independent experiments. Primer annealing temperature for each primer pair was optimized by temperature gradient real-time qRT-PCR and primer efficiency performed for all primers (via a standard curve performed by serial dilution of cDNA). Only primers with an efficiency calculated between 90 and 100% were utilized. Primer sequences and their annealing temperatures are listed in Table 1.

Immunohistochemistry. Kidneys were removed from MRL/lpr mice at early (6–8 wk old) and late (16–18 wk old) disease stages, and from NZM2410 mice at early (8–10 wk old) and late (28–34 wk old) disease stages. The flash-frozen kidneys were embedded in OCT and sectioned at 4- or 5- μm thickness using a cryotome cryostat. Tissue sections were fixed in ice-cold acetone and blocked in 5% BSA. Sections were incubated with rabbit anti-NEU1 or rabbit anti-NEU3 primary antibodies (Santa Cruz Biotechnology). These antibodies were previously shown to specifically detect NEU1 and NEU3, respectively (10), which we confirmed and further determined do not cross-react in a Western immunoblot of extracts from cells transfected with NEU1 and NEU3 expression vectors (data not shown). Sections were incubated with 0.3% H_2O_2 solution in PBS at room temperature to block endogenous peroxidase activity. After washing in PBS, the sections were incubated with HRP-conjugated goat anti-rabbit secondary antibody (Invitrogen, Carlsbad, CA). DAB substrate solution (Vector Laboratories, Burlingame, CA) was applied on tissue sections for 10 min and counterstained in Carazzi's hematoxylin for 45 s. Tissues were dehydrated and cleared in ethanol gradients and xylene and mounted with Cytoseal 60 (Thermo Fisher Scientific, Carlsbad, CA). Staining with secondary alone was used as a negative control. Images were captured on a Nikon Eclipse 90i microscope with a Nikon Digital Sight D5-Fi1 camera and NIS-Elements AR 3.2 soft-

ware at $\times 20$ –40. NEU1 and NEU3 staining within 8–12 glomeruli per section of three mice for each strain at each disease stage were evaluated using the publicly available plugin IHC profiler (50) for scoring DAB staining intensity using ImageJ 1.51m9 software (41). Data presented are the percentage of staining scored as high positive and positive for each glomerulus.

Immunofluorescence. NEU1, NEU3, and HA-IgG were detected in primary MCs using immunolabeling techniques. Cells were allowed to adhere and grow in sterile eight-chamber slide (Fisher, Pittsburgh, PA) for a period of 24 h. The cells were fixed with chilled 4% paraformaldehyde, blocked with 0.5% BSA, for 1 h. HA-IgG was detected with Alexa Fluor 594 goat anti-mouse IgG (Invitrogen, Carlsbad, CA). NEU1 and NEU3 were detected with FITC-conjugated goat anti-rabbit IgG (Southern Biotechnology, Birmingham, AL). Staining with goat anti-rabbit only served as a negative control. Nuclei were counterstained with Hoechst and mounted using Vectashield fluorescence mounting media (Vector Laboratories, Burlingame, CA). Images presented are representative of two experiments with similar results.

NEU1 and NEU3 expression with respect to IgG deposition on renal sections was visualized on kidney sections of MRL/lpr nephritic mice. Sections were fixed in ice-cold acetone, incubated in 10% phosphate-buffered formalin, blocked in 5% BSA, and incubated with rabbit anti-NEU1 or anti-NEU3 antibodies (Santa Cruz Biotechnology). NEU1 and NEU3 were detected with Alexa Fluor 488-conjugated chicken anti-rabbit (Thermo Fisher Scientific), and IgG deposition was detected with Alexa Fluor 594 goat anti-mouse IgG (Invitrogen). Staining with Alexa Fluor 488-conjugated chicken anti-rabbit only served as a negative control. Nuclei were counterstained with Hoechst, mounted, and visualized as performed for the cultured MCs. Staining of renal sections from three MRL/lpr mice were evaluated, and representative images are presented. Images were captured under laser-scanning confocal microscopy with a bright-field overlay on a Nikon Eclipse Ti microscope with an Intensilight C-HGFI Illuminator, Nikon A1r camera, and NIS Elements 4.5 software).

Neuraminidase activity assay. NEU activity in kidney homogenates was measured as described previously using the Amplex Red neuraminidase (Sialidase) assay kit (Invitrogen, Carlsbad, CA) (34) using 250 μg of kidney homogenates from prenephritic (8–9 wk of age) and nephritic (23–26 wk of age) NZM2410 mice. Total protein was determined using the microBCA assay (Thermoscientific, Rockford, IL). Results are reported as relative fluorescence units (RFU) per minute per milligram protein. NEU activity in live cells was measured as follows. Primary MCs were seeded at 20,000 cells per well and cultured (37°C, 5% CO_2) in a black 96-well clear-bottom plate. Cells were treated the following day with vehicle (sterile PBS) or 22.5 $\mu\text{g/ml}$ HA-IgG for 24 h. After incubation, media were collected, and the cells were washed with 37°C warmed sterile filtered Tris-buffered saline (TBS), at pH 7.4. Cells were then incubated at 37°C with 15 μM 2' (4-methylumbelliferyl)- α -D-N-acetylneuraminic acid (4MU-NANA; Sigma Aldrich; St. Louis, MO) substrate in TBS and fluorescence (excitation: 365 nm, emission: 460 nm) was measured over time as indicated. Following incubation, cell lysates were prepared in radioimmunoprecipitation assay (RIPA) buffer and total protein content were found not to vary significantly between treated and untreated wells. Therefore, results are reported as RFU per 20,000 cells.

Statistical analyses. Statistical analyses were performed using GraphPad PRISM 7 software (GraphPad Software, La Jolla, CA). The association of NEU activity between mice with early and late disease (Fig. 1A) or between MCs treated with vehicle and HA-IgG at different time points post hoc treatment (Fig. 4B) were evaluated using a series of two-sample *t*-tests. Adjustment for multiple comparisons for the *P* values in the *t*-tests were calculated using Welch's correction due to unequal variance for Fig. 1A and the Holm-Sidak test for Fig. 4B. The association between IL-6 or MCP-1 levels with HA-IgG concentration and treatment group (empty, NEU1, and

Table 1. Sequences of primers used in real-time PCR

Primer	Sequence
α SMA forward	5'-CCC TGA AGA GCA TCC GAC AC-3'
α SMA reverse	5'-GCC TTA GGG TTC AGT GGT GC-3'
Pdgfr β forward	5'-GTG GTG AAC TTC CAA TGG ACG-3'
Pdgfr β reverse	5'-GTC TGT CAC TGG CTC CAC CAG-3'
Cd31 forward	5'-TAC TGC AGG CAT CGG CAA A-3'
Cd31 reverse	5'-GCA TTT CGC ACA CCT GGA T-3'
Cd144 forward	5'-CAG TGA CAG AGG CCA ATT CT-3'
Cd144 reverse	5'-GCC TCC ACA GTC AGG TTA TAC-3'
Nephrin forward	5'-ACA CAA GAA GCT CCA CGG TTA G-3'
Nephrin reverse	5'-TGG CGA TAT GAC ACC TCT TCC-3'
Neu1 forward	5'-ACG ATG TAG ACA CAG GGA TAG TG-3'
Neu1 reverse	5'-GTC GTC CTT ACT CCA AAC CAA C-3'
Neu3 forward	5'-TTC CAC CTT CCC TTC CTC ATC C-3'
Neu3 reverse	5'-GCA ATA AGC ACC GTT ATC AAC CAT C-3'
β actin forward	5'-AGA TTA CTG CTC TGG CTC CTA G-3'
β actin reverse	5'-CCT GCT TGC TGA TCC ACA TC-3'
Cxcl3 forward	5'-TGT ACC ATG ACA CTC TGC AAC-3'
Cxcl3 reverse	5'-CAA CGA TGA ATT GGC GTG GAA-3'
Cxcl11 forward	5'-GGC TTC CTT ATG TTC AAA CAG GG-3'
Cxcl11 reverse	5'-GCC GTT ACT CGG GTA AAT TAC A-3'
Ifng forward	5'-ATG AAC GCT ACA CAC TGC ATC-3'
Ifng reverse	5'-CCA TCC TTT TGC CAG TTC CTC-3'
Il1a forward	5'-GCA CCT TAC ACC TAC CAG AGT-3'
Il1a reverse	5'-AAA CTT CTC CCT GAC GAG CTT-3'
Il1b forward	5'-GCA ACT GTT CCT GAA CTC AACT-3'
Il1b reverse	5'-ATC TTT TGG GGT CCG TCA ACT-3'
Il6 forward	5'-CTG CAA GAG ACT TCC ATC CAG TT-3'
Il6 reverse	5'-GAA GTA GGG AAG GCC GTG G-3'
Il10 forward	5'-GCT CTT ACT GAC TGG CAT GAG-3'
Il10 reverse	5'-CGC AGC TCT AGG AGC ATG TG-3'

NEU3) were evaluated using two-way ANOVA model, including effects for HA-IgG dose and treatment group (Fig. 2, *B* and *C*). *P* value for all pairwise comparisons between all groups were calculated using a Tukey adjustment, which compares every mean with every other mean. The association between IL-6 and MCP-1 levels with increasing dose of NEU1, NEU3, HA-IgG, or inhibitor at two time points (Figs. 3, 4, *A*, *C*, *D*, 5, *C* and *D*, and 6) or one dose of HA-IgG over time (Fig. 5, *A* and *B*) were evaluated using a repeated-measures two-way ANOVA model. The primary comparisons of interest were active dose/inhibitor with vehicle and time, as indicated in the figures, and the Dunnett adjustment was used, which compares every mean to a control mean. Exact *P* values are provided or are represented as follows: **P* < 0.05; ***P* < 0.005; ****P* < 0.001; *****P* < 0.0001. Type 3 *P* values of main effects for ANOVA models were calculated when appropriate and are provided in the figure legends with post hoc *P* values provided on the graphs.

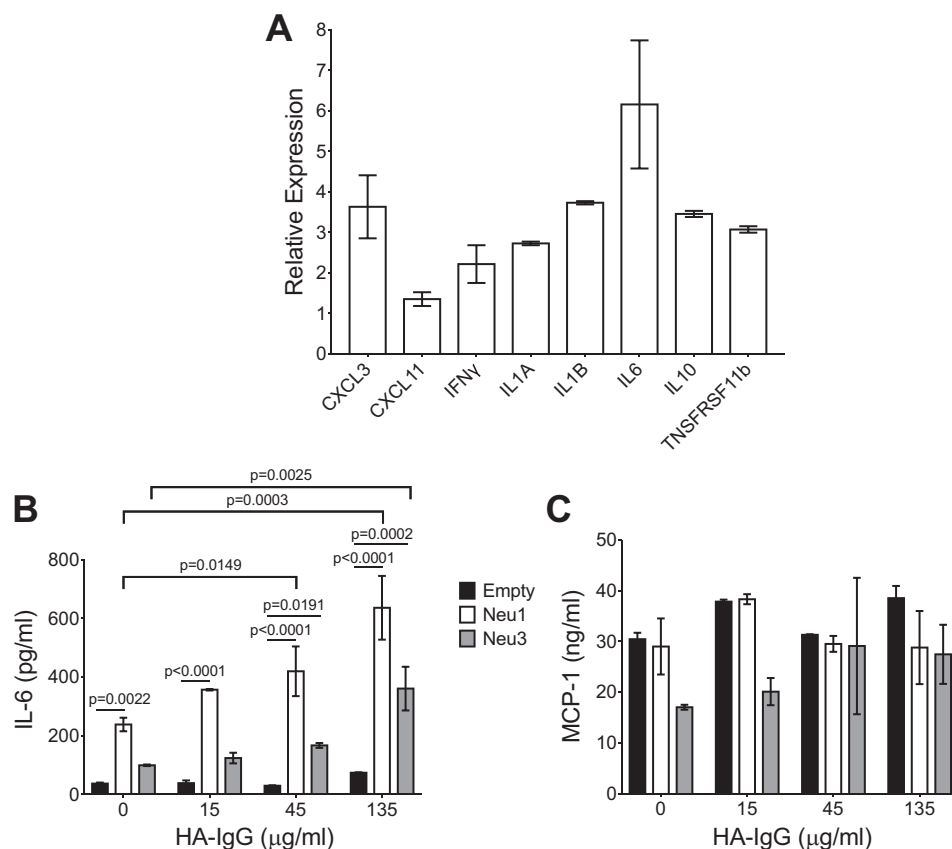
RESULTS

NEU activity and NEU1/NEU3 expression in nephritic kidneys of lupus mice. Previously, we showed that *Neu1* message levels and NEU activity increase in the kidneys of nephritic MRL/lpr lupus mice and parallel increases in LacCer and GlcCer (due to catabolism of gangliosides by NEUs) in the kidneys and urine (34). To determine whether increased NEU activity in the kidneys may be a common phenomenon in lupus nephritis (LN), we measured NEU activity in NZM2410 mice at an early disease stage (8–10 wk old) and a mid- to late-disease stage (23–28 wk old). In agreement with results observed in the MRL/lpr lupus strain, NEU activity is significantly increased in the kidneys of mid- to late-disease stage compared with early disease stage (Fig. 1*A*). Of the eight

23–28-wk-old mice, five had 30–500 mg/dl protein, two had trace amounts of protein, and one was negative for protein in their urine. All of the 8–10-wk-old mice were negative for protein or had only trace amounts of protein in their urine. Although some of the 23–28-wk-old NZM2410 mice had low or no measurable protein in their urine, a hallmark of nephritis, we demonstrated previously in the MRL/lpr lupus strain that renal NEU activity increases as disease progresses and is elevated before significant increases in proteinuria and development of nephritis (34). The results in the NZM2410 mice show the same significant increase in renal NEU activity with disease progression. Together, with our results in LN patients (34), these results further support a potential role of NEU-mediated glycosphingolipid catabolism in LN.

Renal NEU activity is likely due to NEU1 and/or NEU3, which are the two most abundantly expressed NEUs in the kidney (52). Thus, we examined expression of NEU1 and NEU3 in the kidneys of MRL/lpr and NZM2410 lupus mice by immunohistochemistry to determine the renal cell types expressing these factors. The ideal control for the MRL/lpr mice are age-matched MRL/MpJ mice, which develop disease at a much slower rate than the MRL/lpr strain. We previously demonstrated that LacCer, GlcCer, and NEU activity levels are significantly higher in kidneys from age-matched MRL/lpr compared with MRL/MpJ mice and that these levels significantly increase in the MRL/lpr mice as disease progresses (34). Therefore, we compared NEU1 and NEU3 expression in early- and late-disease stage MRL/lpr and NZM2410 mice. Both NEU1 and NEU3 are expressed highly in the glomeruli and are detectable in all glomerular cell types, including mesangial

Fig. 2. GSL catabolism upregulates *IL-6* gene expression and IL-6 production by MES13 MCs. *A*: MES13 MCs were cultured with LacCer and GlcCer, mRNA isolated, and cytokine gene expression was measured by real-time PCR in reverse-transcribed mRNA. Data are an average of two or three independent experiments with gene expression presented as fold increase in LacCer/GlcCer-treated cells over the gene expression in vehicle-treated cells. *B* and *C*: MES13 mouse MCs were transfected with a NEU1, NEU3, or empty expression plasmid in the presence or absence of HA-IgG at increasing concentrations for 24 h. IL-6 (*B*) and MCP-1 (*C*) in media were measured by ELISA and normalized to total protein. The data are representative of three independent experiments with similar results. *P* values were calculated as described in MATERIALS AND METHODS.



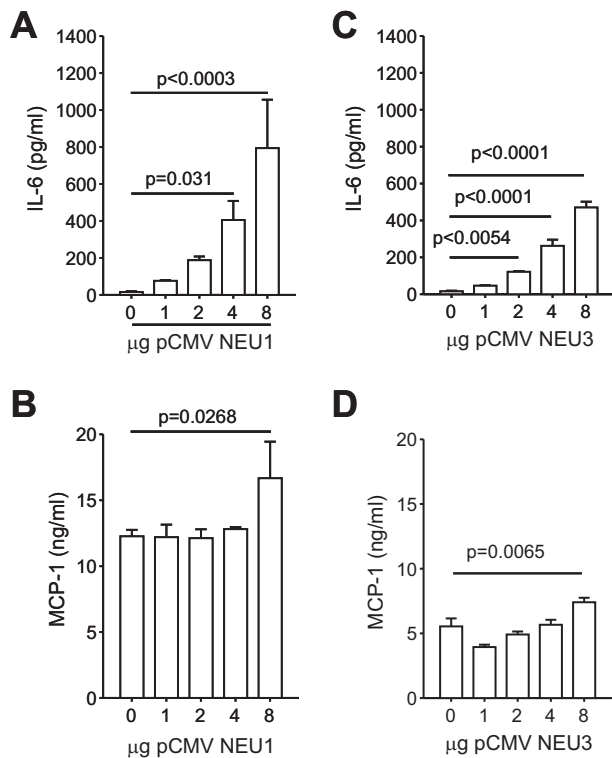


Fig. 3. MES13 MCs produce IL-6 in a dose-dependent manner in response to NEU1 or NEU3 overexpression in the absence of stimulation. MES13 mouse MCs were transfected with increasing concentrations of a NEU1 (A and B) or NEU3 (C and D) expression plasmid and media collected 24 h (not shown) and 48 h (presented) posttransfection. IL-6 (A and C) and MCP-1 (B and D) levels in media were measured by ELISA and normalized to total protein. Data are representative of at least three transfections with similar results. *P* values were calculated as described in MATERIALS AND METHODS. Type 3 main effect *P* value for A and C, $P < 0.0001$. Post hoc *P* values are provided in the graphs.

cells (MCs) (Fig. 1, B and C). Semiquantitation of the staining in glomeruli is shown to the right of the images. NEU1 and NEU3 expression varied from mouse to mouse and among glomeruli within a mouse, especially in the sections from late-disease stage mice of both strains, and showed a trend of increasing NEU1 and NEU3 expression at the late disease stage. Although staining with secondary alone showed slight staining in the sections from late-disease stage mice (likely due to nonspecific interactions with IgG deposits), the NEU1 and NEU3 staining was greater than with secondary alone (data not shown). Together, these results indicate that renal NEU activity increases as disease develops due to increasing expression of NEU1 and/or NEU3. The increased expression may be due to the increase in the number of mesangial cells and/or infiltrating immune cells at the late disease stage. On the basis of the specific staining in MCs at both the early and late disease stages and the important role that MCs play in renal inflammation during LN, we focused our studies on NEU-mediated GSL catabolism in MCs.

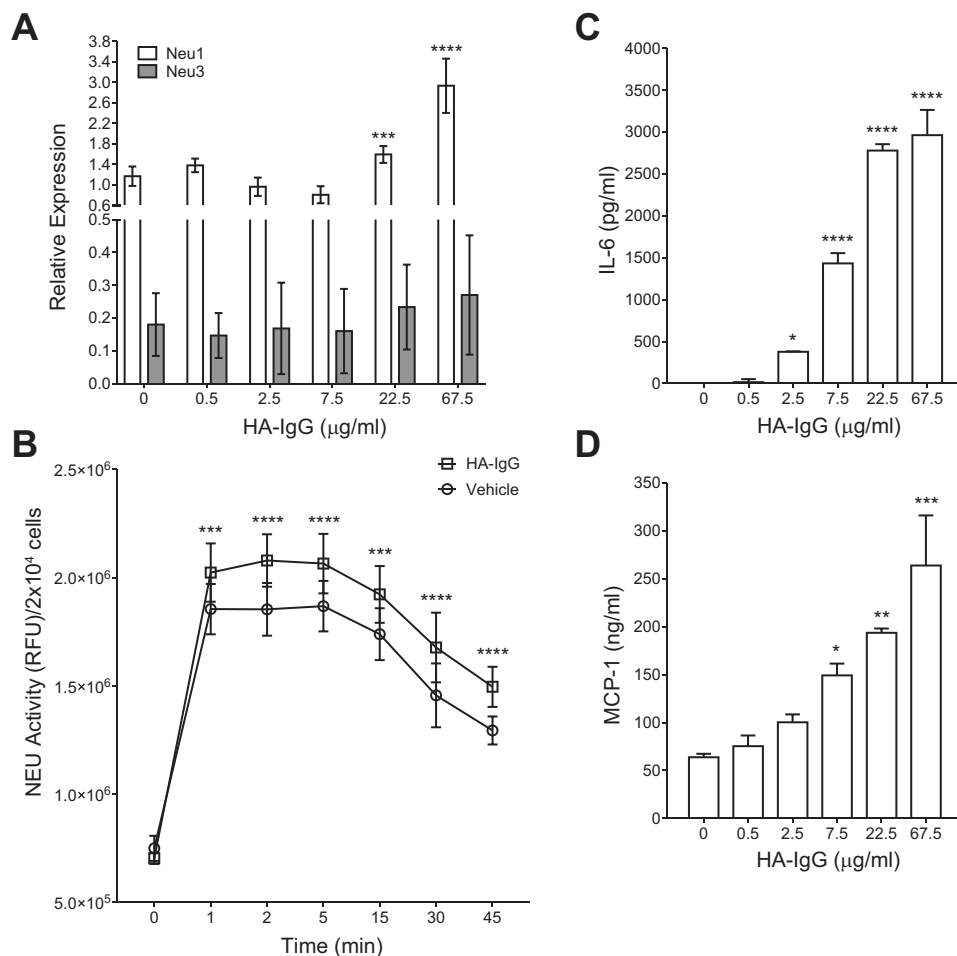
GSL catabolic pathway stimulated MC IL-6 production. An increase in GSL catabolism results in increased LacCer and GlcCer levels, which are elevated in LN (34). To determine the effects of increased LacCer and GlcCer on MCs, we treated the MES13 mouse MC line with short-chain cell-permeable GSL analogs (C8) of GlcCer and LacCer. After treating MES13 mouse MCs with equal amounts of C₈-GluCer and C₈-LacCer,

the expression levels of proinflammatory cytokines and chemokines were determined using a pathway-focused RT² profiler PCR Array (SA Biosciences). Approximately 43 proinflammatory cytokines and chemokines were upregulated (twofold or greater compared with vehicle-treated) in the GSL-treated cells (Table 2). Twelve of 43 genes were identified as kidney-relevant proinflammatory factors using Ingenuity Pathways Analysis software. Six of those genes were verified to be consistently increased twofold or greater (Fig. 2A) using independently designed and optimized primers (Table 1). These results suggest that elevated GSL catabolism in MCs is sufficient to induce production of inflammatory cytokines that play a major role in renal infiltration of immune cells.

IL-6 was one of the more highly upregulated cytokine genes in Fig. 2A with a more than fourfold increase in response to LacCer/GlcCer compared with vehicle. IL-6 acts as an autocrine growth factor for MCs, increasing their proliferation (39), a hallmark of lupus nephritis, and plays an important role as an inflammatory mediator of LN (9, 18). Therefore, we examined the effect of NEU activity on IL-6 production in the MES13 mouse MC line. We also examined effects on MCP-1 production, one of the earliest cytokines produced in LN kidneys following immune complex deposition (47) but was not upregulated in response to LacCer/GlcCer (data not shown). NEU1 and NEU3 are both endogenously expressed in MES13 cells, as detected by immunofluorescence (data not shown). MES13 MCs were treated with heat-aggregated IgG (HA-IgG), a mimic of immune complex deposition, shown to stimulate MCs to produce cytokines (14, 45). However, increasing concentrations of HA-IgG failed to stimulate significant production of IL-6 or MCP-1 in MES13 MCs (Fig. 2B and 2C, black bars). However, overexpressing NEU1 or NEU3 resulted in a significant HA-IgG dose-dependent increase in IL-6 (Fig. 2B), but not MCP-1 (Fig. 2C). Interestingly, both NEU1 and NEU3 significantly and dose-dependently increased the production of IL-6 in the absence of external stimulation by HA-IgG (Fig. 3, A and C). MCP-1 was significantly increased only when transfected with the highest concentrations of NEU1 and NEU3 (Fig. 3, B and D). Data presented are results 48 h after transfection. Similar significant increases were also observed 24 h after transfection (data not shown). Activation of NEU1 requires interaction with protective protein/cathepsin A (PPCA) (8, 49). Since PPCA was not cooverexpressed with NEU1 in these experiments, the IL-6 production in response to NEU1 overexpression may be underestimated. These results are consistent with results with GlcCer/LacCer treatment results and further suggest that GSL catabolism plays an important role in IL-6, but not MCP-1, production.

Activation with HA-IgG increases *Neu1* message levels and NEU activity in MRL/lpr lupus MCs. To determine whether NEU activity plays a role in activation of lupus-prone MCs, we generated primary MC lines from kidneys of early-disease stage (prenephritic) lupus-prone MRL/lpr mice. MCs were treated with different concentrations of HA-IgG (0.5–67.5 μg/ml) for 24 h, and *Neu1* and *Neu3* mRNA expression and NEU activity were measured. HA-IgG significantly increased *Neu1* mRNA expression at the highest concentrations (22.5 and 67.5 μg/ml) (Fig. 4A). Although *Neu3* mRNA also appears to be increasing in response to the higher concentrations of HA-IgG, the increases were not significant. *Neu3* message levels were barely detectable and 2–5-fold lower relative to

Fig. 4. HA-IgG stimulation of primary MRL/lpr lupus-prone MCs increases *Neu1* message levels, NEU activity, IL-6, and MCP-1 production. Primary MRL/lpr MCs were grown out of glomeruli isolated from prenephritic mice and stimulated with HA-IgG. **A**: cells were treated with increasing concentrations of HA-IgG (0.5, 2.5, 7.5, 22.5, and 67.5 $\mu\text{g/ml}$) for 24 h. *Neu1* and *Neu3* mRNA levels were measured in cells by real-time RT-PCR. Results are an average of three independent experiments. **B**: NEU activity was measured in live cells 24 h after cells were treated with 22.5 $\mu\text{g/ml}$ of HA-IgG or vehicle only. Substrate 4MU-NANA was added to cells, and fluorescence was measured at the indicated time points. Results are an average of three independent experiments. **C** and **D**: cells were treated with increasing concentrations of HA-IgG for 24 h. IL-6 (**C**) and MCP-1 (**D**) levels in media were quantified by ELISA and normalized to total protein. Results are representative of at least three independent experiments with similar results. *P* values were calculated as described in MATERIALS AND METHODS. For all graphs, post hoc *P* values are provided on the graphs: **P* < 0.05; ***P* < 0.005; ****P* < 0.001; *****P* < 0.0001. Type 3 main effect *P* values: **A**, *P* < 0.0001; **C**, *P* < 0.0001; **D**, *P* = 0.0008.



Neu1 message levels, which is in agreement with previously published results showing that *Neu3* is less abundant than *Neu1* in the kidney. These results suggest that stimulation with HA-IgG may increase *Neu1* mRNA expression. We then measured NEU activity in live MCs treated with 22.5 $\mu\text{g/ml}$ of HA-IgG, the dose at which we observed a significant increase in *Neu1* mRNA levels. NEU activity was significantly increased in response to HA-IgG treatment, with an immediate significant increase followed by a plateau from 1 to 5 min compared with vehicle-treated cells (Fig. 4B). The increase in *Neu1* message levels and NEU activity corresponded with a significant dose-dependent secretion of IL-6 (Fig. 4C) and MCP-1 (Fig. 4D). Similar dose-dependent increases of IL-6 and MCP-1 were observed 24 h and 48 h after HA-IgG stimulation with the 24-h data presented in Fig. 4. These results suggest that increased NEU activity may be due largely to increased NEU activity following HA-IgG stimulation of lupus-prone MCs. Together, the results in Figs. 2–4 suggest that NEU activity may mediate IL-6 production following activation of lupus-prone MCs.

IL-6 production in HA-IgG-stimulated MRL/lpr lupus MCs is mediated by NEU activity. To determine whether NEU activity plays a role in IL-6 production, we first examined the timing of IL-6 and MCP-1 production following HA-IgG stimulation. IL-6 production increased significantly within 3 h of HA-IgG treatment and reached a plateau from 6 to 48 h

compared with vehicle treatment (Fig. 5A). For MCP-1, a gradual increase in production beginning at 3 h and continuing over 48 h was observed (Fig. 5B). Therefore, we examined the effect of OP on HA-IgG-stimulated IL-6 and MCP-1 production by MRL/lpr MCs. OP is a broad competitive inhibitor of viral NEU activity that also was shown to block mammalian NEU activity in vitro and in vivo (2, 3, 31, 34). OP treatment blocked IL-6 and MCP-1 production in a dose-dependent manner 3 h (data not shown) and 6 h (Fig. 5, C and D) after HA-IgG activation. IL-6 production was reduced 50% at 125 μM OP, whereas 500 μM OP was required to block MCP-1 production by 50%. Because OP inhibits all NEU activity, siRNA *Neu1* was used to knock down *Neu1* message levels in the MRL/lpr MCs. *Neu1* siRNA reduced *Neu1* mRNA levels by ~50–60% (Fig. 5E) and resulted in a significant reduction in IL-6 production (Fig. 5F), but no reduction occurred in MCP-1 production (data not shown) upon activation with HA-IgG. *Neu3* message levels were also measured and were unaffected by *Neu1* siRNA remaining at nearly undetectable levels regardless of treatment (data not shown). These results suggest HA-IgG activation of IL-6 production by lupus-prone MCs is mediated by NEU activity that may be due largely to NEU1 activity.

IL-6 production by lupus serum stimulated MRL/lpr lupus MCs is mediated by NEU activity. Although HA-IgG mimics immune complex deposition, we examined the role of NEU

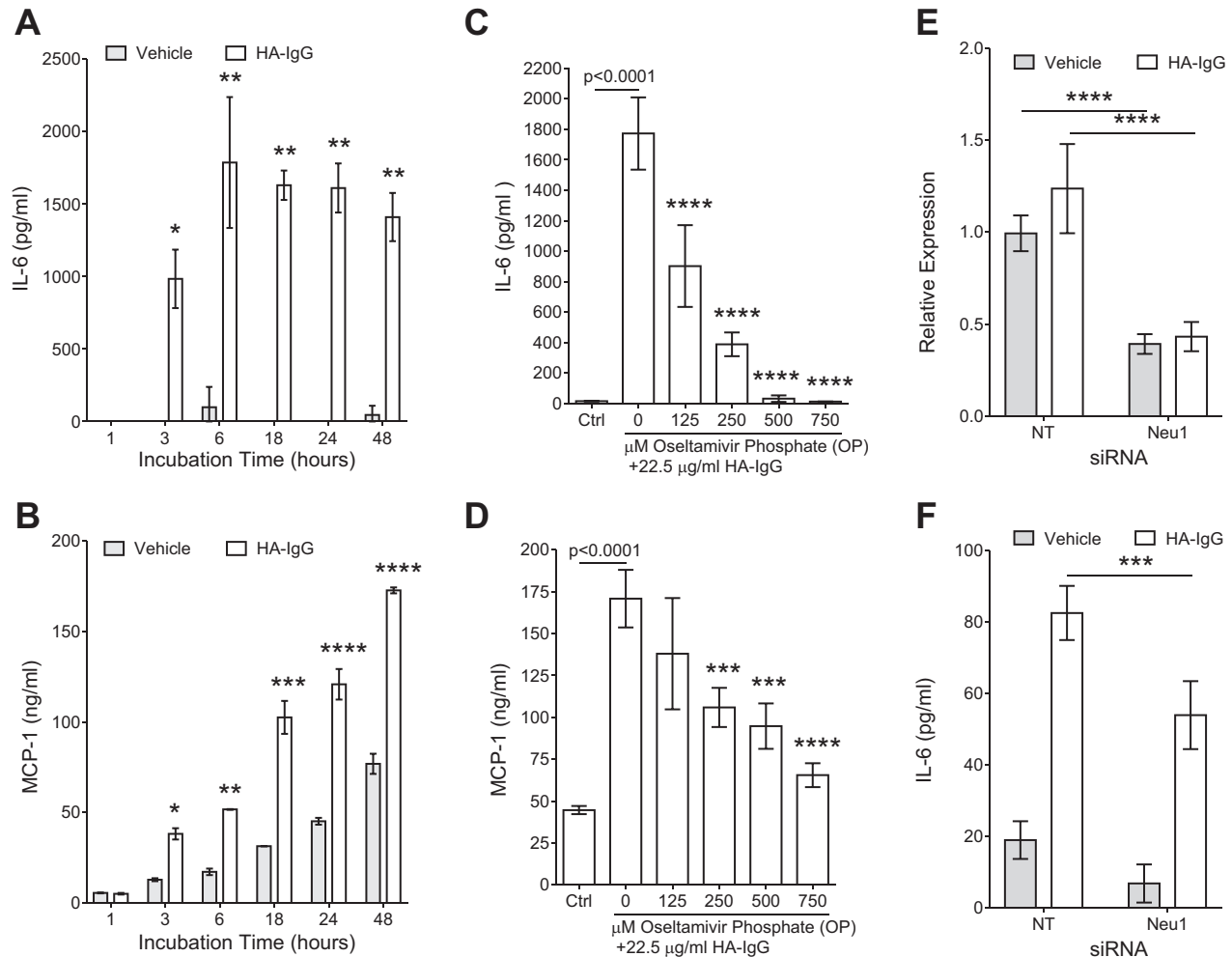


Fig. 5. Inhibiting NEU activity blocks HA-IgG-stimulated IL-6 and MCP-1 production by MRL/lpr lupus prone MCs. **A** and **B**: primary MRL/lpr MCs were treated with 22.5 μ g/ml of HA-IgG over time. IL-6 (**A**) and MCP-1 (**B**) levels were measured in media by ELISA and normalized to total protein. **C** and **D**: primary MRL/lpr MCs were cultured with increasing concentrations of the NEU inhibitor oseltamivir phosphate (OP) for 24 h followed by stimulation with 22.5 μ g/ml of HA-IgG. IL-6 (**C**) and MCP-1 (**D**) levels in the media 6 h (presented) and 3 h (not shown) after stimulation were measured by ELISA and normalized to total protein. **E** and **F**: cells were transfected with *Neu1* siRNA or nontarget siRNA and stimulated with 45 μ g/ml of HA-IgG for 24 h and *Neu1* mRNA levels in cells (**E**) and IL-6 production in media (**F**) were measured. All results are representative of at least three independent experiments with similar results. For all graphs, post hoc *P* values are provided on the graphs: **P* < 0.05; ***P* < 0.005; ****P* < 0.001; *****P* < 0.0001. Type 3 main effect *P* values: **A**, *P* = 0.0026; **B**, *P* < 0.0001; **C**, *P* < 0.0001; **D**, *P* = 0.0018.

activity in IL-6 and MCP-1 production in the primary MCs when stimulated with serum from 18-wk-old nephritic MRL/lpr mice, which contains high levels of IgG (53). IL-6 production by the MCs increased as the percentage of MRL/lpr serum increased, which was significant with 10% lupus serum (Fig. 6A). Serum from C57BL/6 (B6) healthy mice had no effect. Neither MRL/lpr serum (5 to 20%) nor B6 serum (20%) resulted in significant production of MCP-1 (Fig. 6B). Importantly, as with HA-IgG stimulation, OP significantly suppressed lupus serum-stimulated IL-6 production in a dose-dependent manner 3 h (Fig. 6C) and 6 h (data not shown) after stimulation. These results indicate that IL-6 production of lupus prone MCs following activation by lupus serum is mediated by NEU activity. Combined with the results above with HA-IgG, it is likely that NEU may specifically mediate IL-6 production by lupus-prone MCs activated by circulating IgG-containing immune complexes.

Expression of NEU1 and NEU3 overlap with IgG deposits in MRL/lpr MC cultures and renal sections. NEU3 is typically located in the plasma membrane and although NEU1 is largely located in the lysosome, it is also found at the cell surface in many cell types (23, 25, 33). Therefore, to determine whether NEU1 and/or NEU3 may be colocalizing with HA-IgG binding on the surface of the MRL/lpr MCs, we performed immunofluorescence. As demonstrated previously in B6 MCs (45), HA-IgG binds to the surface of MRL/lpr MCs (Fig. 7B). Staining for both HA-IgG and either NEU1 (Fig. 7C) or NEU3 (Fig. 7D) shows that HA-IgG binding overlaps with cell surface staining for NEU1 and NEU3. We then examined localization of NEU1 and NEU3 with respect to IgG deposition in renal sections from nephritic MRL/lpr mice. Similar to the overlap observed in the MC cultures, overlap was observed between IgG deposits (red) and either NEU1 (Fig. 1E) or NEU3 (Fig. 7F) (green) in the glomeruli specifically in the

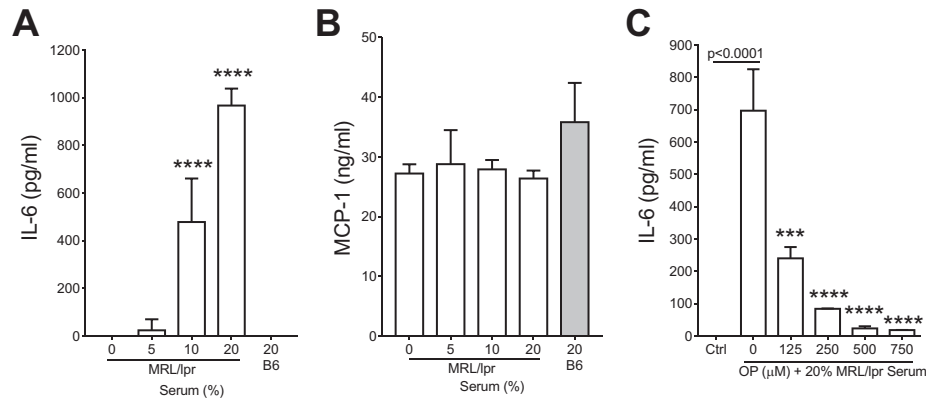


Fig. 6. Inhibiting NEU activity blocks lupus serum-stimulated IL-6 production by MRL/lpr lupus prone MCs. Primary MRL/lpr MCs were treated with increasing concentrations of MRL/lpr serum or B6 serum for 24 h. IL-6 (A) and MCP-1 (B) levels in media were measured by ELISA and normalized to total protein. C: MRL/lpr MCs were cultured with increasing concentrations of OP for 24 h followed by stimulation with 20% MRL/lpr lupus serum. IL-6 levels were quantified 3 h (not shown) and 6 h (presented) after stimulation by ELISA and normalized to total protein. All results are representative of at least three independent experiments with similar results. For all graphs, post hoc *P* values are provided on the graphs: ****P* < 0.001; *****P* < 0.0001. Type 3 main effect *P* values: A, *P* < 0.0001; C, *P* < 0.0001.

mesangium (arrows). Although the extent of overlap varied among glomeruli, only occasional and faint overlap was observed between NEU1 and IgG, whereas NEU3 and IgG overlap was stronger and present in most of the glomeruli. The close proximity of NEU1 and NEU3 with HA-IgG binding in MCs and with IgG deposits in the mesangium of renal sections suggests that NEU1 and/or NEU3 may be present in a complex containing IgG deposits in mesangial cells of nephritic mice to mediate downstream effects of IgG deposition in the kidney.

DISCUSSION

We previously reported that NEU activity and *Neu1* mRNA levels are elevated in the kidney of nephritic mice compared with their nonnephritic counterparts (34). We have now demonstrated that NEU activity in nephritic NZM2410 lupus mice also is significantly increased compared with prenephritic NZM2410 mice, indicating that increased renal NEU activity likely is a common phenomenon in lupus mouse strains that is translationally relevant to human lupus. Of the four mammalian *Neu* genes, *Neu1* and *Neu3* are the most highly expressed in the kidney (7, 28–30), and we showed that both NEU1 and NEU3 proteins are readily detected in the glomeruli, including mesangial cells of healthy and nephritic mice. It remains to be determined whether the increased NEU activity detected in the MRL/lpr (34) and NZM2410 (Fig. 1A) kidneys is a result of increased NEU1/NEU3 expression and/or activation.

MCs occupy the central stalk of the glomerulus and interact with endothelial cells and podocytes. It gives structural support and contributes to the regulation of glomerular filtration, occupying ~40% of the total cells in the glomerulus. MCs exhibit both innate and adaptive immune properties, such as cytokine production and phagocytosis and are important early drivers in promoting renal inflammation in LN. Their response to pathological insult is associated with key events in glomerular dysfunction. Activating MCs by various methods leads to production of a number of proinflammatory cytokines and chemokines, including IL-6 and MCP-1. Here, we demonstrated that increasing LacCer and GlcCer levels in the MES13 MC line upregulates a number of cytokine genes, including *Il-6*. IL-6 plays an important role in the progression of lupus

nephritis. Lupus mice treated with anti-IL-6 antibodies or with a homozygous knockout of IL-6 had significantly improved survival and reduced/delayed nephritis that included reduced inflammatory cell infiltration (9, 23). Of translational significance, LN patients with active disease were shown to have increased renal and urinary IL-6 levels, with both MCs and inflammatory cells contributing to elevated renal levels (13, 16, 18, 36, 46, 48). The elevated renal IL-6 levels are thought to be largely due to infiltrating inflammatory cells in LN patients. However, on the basis of studies of IL-6 in lupus mouse strains (9, 19, 22), IL-6 produced by MCs appears to play a role in promoting the production of other cytokines in the kidney to attract inflammatory cells following immune complex deposition early in disease, which is difficult to study in human patients.

Here, our studies focused on IL-6 production following activation of lupus-prone MRL/lpr MCs using heat-aggregated IgG (HA-IgG) shown to bind and activate MCs, mimicking immune complex deposition (14, 37, 38, 45), an early event in LN. In this report, we demonstrated that *Neu1* expression, NEU activity, and IL-6 and MCP-1 production are significantly increased in lupus-prone MCs following activation. Overexpression studies of NEU1 or NEU3 in the mouse MC line MES13, inhibition of NEU activity in activated lupus-prone MCs using the FDA-approved drug OP, and overlapping expression of NEU1/NEU3 with HA-IgG binding at the cell surface support a role for NEU activity in mediating a signaling pathway leading to IL-6 production following immune complex activation of lupus-prone MCs. The stimulation of IL-6 production, but not MCP-1, by LacCer/GlcCer and NEU1/NEU3 in the MES13 MCs, the more pronounced effect on IL-6 production compared with MCP-1 when NEU activity is blocked by OP, and the decrease in IL-6 but not in MCP-1 in activated cells treated with *Neu1* siRNA strongly suggests that GSL catabolism (NEU activity) directly impacts IL-6 production in activated lupus-prone MCs.

The inhibitor studies using the NEU activity inhibitor OP with either HA-IgG- or lupus serum-activated lupus-prone MCs demonstrated that inhibiting NEU activity blocks IL-6 production. Although this drug was developed to inhibit viral

Table 2. Cytokine and chemokine genes upregulated twofold or more in MES13 MCs in response to LacCer and GlcCer treatment in a PCR array

Gene Symbol	Fold Regulation
<i>Adipoq</i> *	5.44
<i>Ccl1</i>	6.25
<i>Ccl12</i>	4.07
<i>Ccl22</i>	3.93
<i>Ccl24</i>	5.29
<i>Ccl3</i>	4.71
<i>Ccl4</i>	4.01
<i>Cd40lg</i>	10.02
<i>Cd70</i>	6.47
<i>Csf2</i>	3.57
<i>Cxcl13</i>	3.06
<i>Cxcl3</i> *	3.57
<i>Cxcl9</i> *	4.13
<i>Cxcl11</i> *	2.27
<i>Fasl</i>	3.80
<i>Hc</i>	9.09
<i>Ifna2</i>	3.59
<i>Ifng</i> *	5.71
<i>Il10</i> *	8.66
<i>Il12a</i>	4.01
<i>Il12b</i>	7.38
<i>Il13</i>	3.80
<i>Il16</i>	8.84
<i>Il17a</i> *	3.42
<i>Il1a</i> *	2.27
<i>Il1b</i> *	3.67
<i>Il2</i> *	4.67
<i>Il21</i>	6.52
<i>Il22</i>	5.04
<i>Il3</i>	3.82
<i>Il4</i>	4.77
<i>Il5</i>	4.97
<i>Il6</i> *	4.15
<i>Mstn</i>	4.94
<i>Nodal</i>	6.89
<i>Osm</i>	3.19
<i>Pf4</i>	5.67
<i>Thpo</i>	3.77
<i>Tnf</i>	7.75
<i>Tnfrsf11b</i> *	4.91
<i>Tnfsf10</i> *	4.18
<i>Tnfsf13b</i>	4.07

*Kidney-relevant upregulated cytokines/chemokines.

NEU activity, it was shown to inhibit mammalian NEU1 and NEU3 activity (15, 31). We observed a 50% reduction in IL-6 production at 125 μ M OP, whereas 500 μ M OP was needed to block MCP-1 by 50%. Experiments using lower concentrations of OP were performed, but significant reduction of IL-6 production was not observed below $\sim$ 100 μ M OP (data not shown). We also tested the NEU activity inhibitors Zanamivir (Relenza) and 2-deoxy-2,3-didehydro-*N*-acetylneuraminic acid (DANA), both of which preferentially inhibit NEU2, NEU3, and NEU4 (15, 26, 54). Zanamivir and DANA failed to block IL-6 production following HA-IgG activation of the lupus-prone MCs using millimolar levels of the two inhibitors (data not shown). HA-IgG activation of lupus MCs significantly increased *Neu1* and not *Neu3* message levels, yet overexpression of either NEU1 or NEU3 resulted in significant IL-6 production in the mouse MC line MES13. These results suggest that NEU1 and NEU3 may have overlapping or compensatory functions with respect to IL-6 production. Although

these collective results are suggestive of a NEU1-specific mediated pathway leading to IL-6 production in the MRL/lpr MCs, additional studies are required to definitively determine which NEU is primarily responsible.

Although the increase in renal NEU activity appears to be due to an increase in NEU1 and/or NEU3 expression, the increased expression may be due to a greater number of mesangial cells and/or infiltrating immune cells at the late disease stage. In the culture MCs, the increase in *Neu1* message was only observed at the higher doses of 22.5 and 67.5 μ g/ml HA-IgG, while significant increases in IL-6 were observed beginning at 7.5 μ g/ml HA-IgG. Our results with OP, which is expected to inhibit NEU activity at the plasma membrane only, and the live cell NEU activity assay that measures activity at the cell surface, support a mechanism by which NEU1/NEU3 activity is increased at the plasma membrane and/or a change in cellular location of NEU1 from the lysosomal compartment to the plasma membrane when the MCs are stimulated.

Blocking IL-6 production by OP required treatment of the cells with OP before HA-IgG activation. NEU1 was shown to have broader substrate specificity compared with the other NEUs (43); however, NEU1 and NEU3 exhibit some overlapping specificities. NEU1 preferentially desialylates glycoconjugates in the lysosome and glycopeptides, including cell surface receptors, in the plasma membrane (11, 25, 40). Both NEUs desialylate glycolipids (gangliosides) with NEU3, exhibiting greater specificity than NEU1 for gangliosides (40). Because loss of sialic acids, and specifically loss of ganglioside GM3, increases MC proliferation (20), we speculate that inhibiting NEU activity before activation of the cells may allow time for gangliosides to accumulate (and/or LacCer/GlcCer levels to decrease), resulting in decreased MC activation and hence, IL-6 production. Alternatively, inhibition of NEU activity may block IL-6 production by increasing sialylation levels of a receptor in the IL-6 signaling pathway based on reports that NEU1 can remove sialic acids from cell surface receptors to regulate downstream signaling (6, 17).

NEU3 is primarily located in the plasma membrane, whereas NEU1 can be found located both in the lysosome and plasma membrane (23, 25, 29, 33). Our immunofluorescence results in nonpermeabilized and permeabilized lupus-prone primary MCs and in the MES13 MC line showed that NEU1 is located both intracellularly and at the cell surface (Fig. 7 and data not shown). Results in MRL/lpr primary MC cultures demonstrated that NEU1 and NEU3 expression overlaps with HA-IgG binding. Importantly, NEU1 and NEU3 expression was also observed to overlap with IgG deposits in the mesangium of glomeruli in renal sections from nephritic MRL/lpr mice. In general, more overlap was observed between NEU3 and IgG. This may be a result of NEU3 being more prominent at the cell surface than NEU1, which is predominantly located in the lysosomes with some cell surface expression depending on the cell type (23, 25, 33). Alternatively, the extent of NEU1 or NEU3 overlap with IgG may change depending on disease stage. We may observe more or less overlap at an earlier stage of disease with initial immune complex deposition. These results suggest NEU1 and NEU3 may be part of a cell surface complex containing IgG and play a role in downstream signaling and IL-6 production.

NEUs can interact with and activate Fc receptors (FcRs) (42), which were shown to be essential for disease initiation in

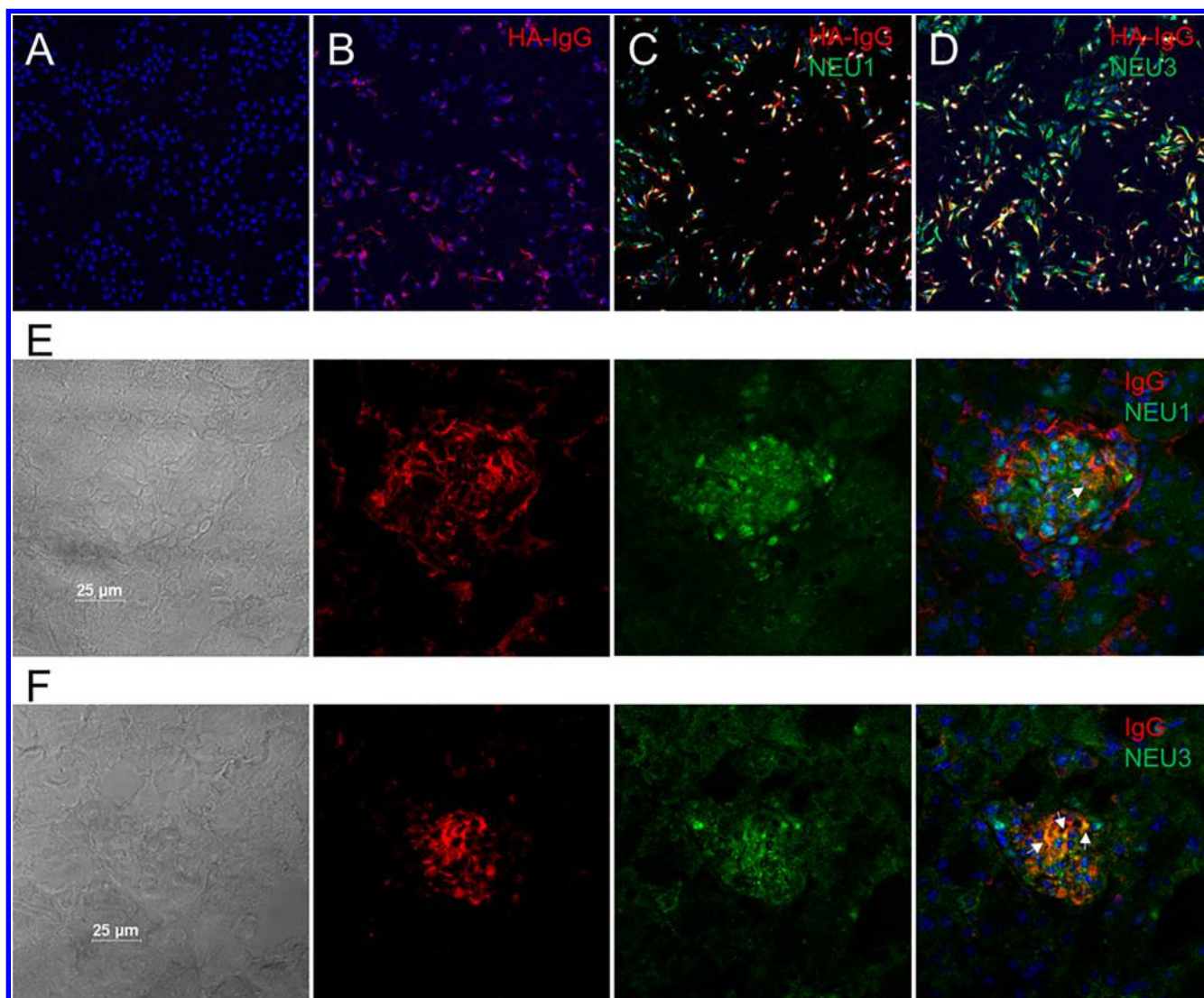


Fig. 7. NEU1 and NEU3 expression overlaps with IgG deposits on the surface of MRL/lpr lupus-prone MCs and in the glomeruli of nephritic MRL/lpr mice. MRL/lpr lupus-prone MCs were fixed (but not permeabilized) and stained with isotype (A) or HA-IgG for 1 h followed by Alexa Fluor 594 goat anti-mouse IgG secondary antibody (B–D). NEU1 (C) and NEU3 (D) were detected using rabbit anti-NEU1 and anti-NEU3 primary antibodies, respectively followed by a FITC-conjugated anti-rabbit secondary. Staining was not observed with secondary antibodies alone (not shown). HA-IgG, red; NEU1 and NEU3, green; nuclei, blue. Results are representative of two independent experiments. E and F: renal sections from nephritic MRL/lpr mice were stained for IgG deposition with Alexa Fluor 594 goat anti-mouse IgG secondary antibody and for NEU1 (E) or NEU3 (F), as indicated above. Bright-field and fluorescent images for IgG (red) and NEU1 or NEU3 (green) are presented independently, and then a composite image of IgG, NEU1, or NEU3, and nuclei staining (blue) is presented. White arrows indicate areas of overlap, which are largely mesangial, between IgG and NEU1 or NEU3 in the composite images.

response to immune complex deposition in LN (5). MCs express FcRs that bind IgG, and HA-IgG was shown to colocalize with Fc γ receptors (Fc γ R) III or IV on MCs and trigger production of inflammatory mediators, including IL-6 and MCP-1 (37, 38, 45). However, studies with Fc γ R-deficient MRL/lpr mice suggest that development of nephritis in this strain is independent of Fc γ R (27). Immune complexes also can signal through Toll-like receptors (TLRs), which are expressed by MCs and have been implicated in the development of LN (12, 21, 24, 35, 44). NEU1 was shown to colocalize with TLR2 and TLR4 on the surface of macrophages and dendritic cells and mediate signaling (1, 4, 10). TLRs 1–4 are expressed by cultured MRL/lpr MCs (35). Alternatively, NEU activity may mediate IgG activation of MCs through an unknown receptor complex or through IgG receptor-independent effects.

Our NEU1 and NEU3 overexpression results in the MES13 MCs suggest that NEU activity can activate an IL-6 signaling pathway in the absence of exogenous stimulant.

In conclusion, we demonstrated that 1) overexpression of NEU1 or NEU3 alone or in conjunction with HA-IgG stimulates significant IL-6 production by the MES13 mouse MC line; 2) HA-IgG activation of lupus-prone primary MCs significantly increased *Neu1* message levels and NEU activity; 3) NEU activity mediates IL-6 production in response to HA-IgG or lupus serum; and 4) NEU1 and NEU3 expression overlaps with IgG binding at the cell surface of lupus-prone primary MCs, and NEU3 expression overlaps with IgG deposits in renal sections of nephritic lupus mice. Taken together, our results reveal a novel NEU-mediated IL-6 pathway in lupus-prone MCs that may promote LN in response to MC activation.

Additional studies are ongoing to identify the molecular mechanisms by which NEU activity mediates IL-6 production in healthy and lupus-prone MCs.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

K.S. and T.K.N. conceived and designed research; K.S., J.I.R., S.M., and T.K.N. performed experiments; K.S., J.I.R., S.M., L.J.S., E.B., and T.K.N. analyzed data; K.S., L.J.S., E.B., and T.K.N. interpreted results of experiments; K.S., J.I.R., and T.K.N. prepared figures; K.S. drafted manuscript; K.S., J.I.R., S.M., L.J.S., E.B., and T.K.N. approved final version of manuscript; J.I.R., K.S., and T.K.N. edited and revised manuscript.

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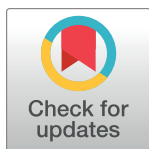
RESEARCH ARTICLE

Targeting glycosphingolipid metabolism as a potential therapeutic approach for treating disease in female MRL/lpr lupus mice

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Abstract

Glycosphingolipids (GSLs) hexosylceramides and lactosylceramides are elevated in lupus mice and human patients with nephritis. Whereas other renal diseases characterized by increased GSL levels are thought to be a result of upregulated GSL synthesis, our results suggest elevated hexosylceramides and lactosylceramides in lupus nephritis is a result of increased catabolism of ganglioside GM3 due to significantly increased neuraminidase (NEU) activity. Thus, we hypothesized GM3 would be decreased in lupus nephritis kidneys and blocking NEU activity would reduce GSLs and improve disease in lupus mice. Female MRL/lpr lupus mice were treated with water or the NEU inhibitor oseltamivir phosphate at the onset of proteinuria to block GSL catabolism. Age-matched (non-nephritic) female MRL/MpJ lupus mice served as controls. Renal GM3 levels were significantly higher in the nephritic MRL/lpr water-treated mice compared to non-nephritic MRL/MpJ mice, despite significantly increased renal NEU activity. Blocking GSL catabolism increased, rather than decreased, renal and urine GSL levels and disease was not significantly impacted. A pilot study treating MRL/lpr females with GlcCer synthase inhibitor Genz-667161 to block GSL synthesis resulted in a strong significant negative correlation between Genz-667161 dose and renal GSL hexosylceramide and GM3 levels. Splenomegaly was negatively correlated and serum IgG levels were marginally correlated with increasing Genz-667161 dose. These results suggest accumulation of renal GM3 may be due to dysregulation of one or more of the GSL ganglioside pathways and inhibiting GSL synthesis, but not catabolism, may be a therapeutic approach for treating lupus nephritis.

Introduction

Systemic Lupus Erythematosus (SLE or lupus) is an autoimmune disease characterized by the development of autoantibodies, immune complex formation and deposition in target organs,

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and subsequent inflammation and organ damage. Glomerulonephritis, the most severe complication of lupus, affects up to 70% of lupus patients and is associated with high morbidity and mortality. Although there has been a 30% reduction in mortality of lupus nephritis (LN) patients with end-stage renal disease (ESRD) over the past 20 years [1], current therapies for treating LN are effective only in a small percentage of patients with 20–30% of patients progressing to ESRD [2–4]. New therapies are desperately needed for treating lupus patients with nephritis to prevent ESRD. The features of LN are similar to other chronic kidney diseases characterized by altered glycosphingolipid (GSL) metabolism, including diabetic nephropathy (DN) [5, 6], polycystic kidney disease (PKD) [7–9], and nephritis associated with aging [10]. Enriched in the kidney, GSLs can play a pathogenic role in progression of renal disease [6, 7, 11, 12].

We previously demonstrated GSL metabolism is altered in both lupus patients and lupus mice with nephritis. Specifically, glucosylceramides (GlcCers), lactosylceramides (LacCers), and neuraminidase (NEU) activity are elevated in the kidneys and urine of MRL/lpr and NZM2410 lupus mice with nephritis and in urine of lupus patients with nephritis compared to their non-nephritic counterparts and healthy controls [13, 14]. Unlike, DN and PKD where elevated GlcCer/LacCer appears to be due to increased GSL synthesis [5, 7, 8], our previous results suggested elevated NEU-mediated GSL catabolism may be responsible for elevated LacCer and GlcCer. NEUs remove terminal sialic acids from glycolipids, including gangliosides such as GM3, and glycoproteins. In the kidney, GSLs play an important role in the proper function of mesangial cells and podocytes [14–17]. Therefore, we hypothesized elevated LacCers/GlcCers and loss of sialic acids, specifically from gangliosides, due to increased NEU activity in the kidney, may contribute to progression of LN.

Based on our previous studies, we measured the levels of additional lipids including ganglioside GM3, the precursor for other sialic acid-containing gangliosides [18], in the kidneys of female MRL/lpr lupus mice and their non-nephritic counterparts (MRL/MpJ). We also tested if blocking NEU activity in MRL/lpr female mice would correct altered GSL metabolism and improve disease. Nephritic MRL/lpr mice showed elevated levels of renal GM3 compared to age-matched MRL/MpJ mice and MRL/lpr mice treated with the NEU inhibitor resulted in a further trend of increased GM3 levels. Unexpectedly, NEU inhibitor treatment slightly increased GSLs and failed to significantly impact renal or immune disease measures in the MRL/lpr mice. We then performed a pilot treatment study in MRL/lpr mice with an inhibitor of GlcCer synthase, which resulted in a significant negative correlation between inhibitor dose with renal GSLs levels and splenomegaly. There was also a trend towards lower serum IgG levels at the higher doses of the inhibitor. Together these results demonstrate GM3 accumulation in MRL/lpr nephritic mice despite increased elevated renal NEU activity, and blocking the synthesis pathway may normalize renal GSL metabolism and reduce disease measures.

Materials and methods

Ethics statement and mouse strains

All animal studies and methods of euthanasia were approved by the local Institutional Animal Care and Use Committee (IACUC). The oseltamivir phosphate study was also approved by the Department of Defense IACUC. Breeding pairs of MRL/lpr mice were purchased (Stock #000485, Jackson Laboratory, Bar Harbor, ME) and bred in-house to obtain female littermates for all studies. Breeding pairs of MRL/MpJ mice were purchased (Stock #000486, Jackson Laboratory, Bar Harbor, ME) and bred in-house to obtain female littermates. All mice used in the presented studies were female. Mice were maintained on a 12 h light-dark cycle in groups of 3–5 per cage in a pathogen free environment with access to food and water ad libitum

throughout the study. Once a week, mice were housed individually in metabolic cages to collect urine during the study as described below after which time they were returned to their same group housing. Mice exhibiting respiratory distress due to gavage, or signs of advanced disease including skin rash over greater than 10% of their body surface, >15% loss of body weight, or decreased grooming prior to study endpoints were euthanized.

Oseltamivir phosphate treatment study

Beginning at 8 weeks of age, 30 MRL/lpr female mice were monitored bi-weekly for onset of proteinuria using Chemstrip 7 protein strips (Roche, Indianapolis, IN). When at least half of the mice showed measurable proteinuria (12–13 weeks of age), mice were divided into oseltamivir phosphate (OP)- and water-treatment groups with equivalent numbers of mice with high and low levels of proteinuria in each group (based on protein strip estimates). Mice administered OP at 10 mg/kg/day have similar plasma levels of oseltamivir carboxylate, the active metabolite of OP, with the same area under the curve (AUC) as humans administered OP at 75 mg/kg twice daily [19]. Moreover, a 10 mg/kg dose in mice was effective in blocking mammalian neuraminidase activity *in vivo* [20, 21]. Therefore, mice received vehicle (water) or 10 mg/kg/day oseltamivir phosphate (OP) (Santa Cruz Biotechnology sc-208135A; Dallas, TX) by gavage daily for four weeks. The study was initiated with 16 mice in the water treatment group and 15 mice in the OP treatment group. One mouse in the water treatment group was euthanized during the first week of treatment due to complications from gavage and removed from the study. Hence, there were 15 MRL/lpr mice in each treatment group. One mouse from each group died and one mouse from each group was euthanized prior to the end of the study. All mice that died or were euthanized had urine albumin >50 mg/day for at least a week and enlarged lymph nodes. Renal samples from the euthanized mice were included in the endpoint analyses and results showed pathological changes consistent with renal disease for both mice. Thus, we concluded the four study mice succumbed or were succumbing due to advanced disease. Gavage was performed at the same time of day for all mice throughout the four-week treatment period. Treatments were performed in two successive sets of mice (6–8 mice per treatment group) due to constraints on metabolic cage availability and personnel for performing gavage. Ten female MRL/MpJ littermates were used as age-matched controls and remained untreated. Twenty-four-hour (24 h) urine and serum were collected one day prior to beginning treatment and then weekly throughout the study. Body weight was monitored throughout the study. Mice were euthanized and organs immediately harvested for analyses. One kidney was flash frozen and one was fixed and paraffin embedded. Approximately one-fourth of the spleen was used for flow cytometry and the rest was flash frozen and stored.

Genz-667161 treatment study

Fifteen female MRL/lpr littermates were monitored bi-weekly beginning at 8 weeks of age for onset of proteinuria using Chemstrip 7 protein strips (Roche, Indianapolis, IN). When at least half of the mice showed measurable proteinuria (12–13 weeks of age), the mice were randomly assigned to a treatment with three mice per group and their food switched to the same chow containing Genz-667161, a glucosylceramide synthase inhibitor developed by Sanofi Genzyme (Waltham, MA). The drug was formulated into the chow by Harlan Laboratories (Madison, WI) such that the mice received approximately 0, 1, 3, 10, or 20 mg/kg/day for four weeks. Twenty-four-hour (24 h) urine and serum were collected one day prior to beginning treatment and then weekly throughout the study. Body weight was monitored throughout the study. At completion of the study, mice were euthanized and organs removed. The spleen was weighed

and flash frozen, one kidney was flash frozen, and one kidney was fixed and paraffin embedded.

Neuraminidase activity assay and lipid measures in kidney homogenates, urine, or mesangial cells

Frozen kidney cortex tissue was homogenized in 20 mM Tris HCl pH 7.4 buffer containing protease inhibitors (Roche, Indianapolis, IN) as described previously [14]. Protein concentration was measured using the BCA assay. Renal neuraminidase activity of kidney homogenates (250 µg) was measured using Amplex Red Neuraminidase Assay Kit according to manufacturers' protocol (Invitrogen A22178; Carlsbad, CA). Mesangial cell neuraminidase activity was measured in live cells as follows. Cells were washed with 37°C warmed sterile filtered Tris-buffered saline (TBS) pH 7.4 and incubated with 15 µM substrate 2' (4-methylumbelliferyl)- α -D-N-acetylneuraminic acid (4MU-NANA) in 37°C warmed sterile filtered TBS (Sigma Aldrich; St. Louis, MO). Fluorescence intensity (excitation: 365 nm, emission: 460 nm) was measured on a SpectraMax i3c fluorometer with SoftMax Pro 6 software (Molecular Devices, San Jose, CA) at baseline prior to substrate addition and in the presence of substrate. Relative fluorescence units (RFU) per 2×10^4 cells after 45 min incubation with substrate are presented on the graph. Total protein content was consistent between untreated and treated cells. Ceramides (Cers), hexosylceramides (HexCers), lactosylceramides (LacCers), sphingomyelins (SMs), Sphingosine-1 Phosphate (S1P), Sphingosine (So), Dihydrosphingosine-1 Phosphate (DHS1P), or Dihydrosphingosine (DHSo) were quantified in kidney homogenates (1 mg), 24-hr urine collections (100 µl), or mesangial cells (1×10^6 cells) by the Virginia Commonwealth University (VCU) Lipidomics/Metabolomics Core Facility or Medical University of South Carolina Lipidomics Core Facility, and Ganglioside GM3s were quantified by the VCU Lipidomics Facility as described previously [13, 22]. Lipid levels in urine were normalized to urine creatinine. Since the OP study was performed in two sets, relative levels for each GSL were calculated within each set of mice, sets were combined, and presented as fold change.

Urine albumin and creatinine ELISA

Urine was collected over a 24 h period using individual metabolic cages once a week. Urine was collected into tubes containing an antibiotic cocktail of Ampicillin, Gentamicin, and Chloramphenicol to prevent bacterial growth. Total albumin levels were measured by ELISA as previously described [13]. Briefly, wells were pre-coated overnight at 4°C with goat anti-mouse Albumin (Fitzgerald Industries, Acton, MA; Cat# 70R-AG001) in 0.05M Carbonate Bicarbonate, blocked with PBS-Tween20 (PBST) + 0.25% gelatin (PBST-GT) and washed with (PBST). Mouse albumin standard (Sigma-Aldrich, St. Louis, MO; cat# A-3559) and urine were serially diluted with PBST-GT directly in the plate and incubated. After washing with PBST, albumin was detected with HRP-conjugated goat-anti-mouse Albumin (Fitzgerald Industries, Acton, MA; Cat# 60R-AG002) and 3,3',5,5'-tetramethylbenzidine (TMB, Sigma, St. Louis, MO) in citrate buffer (3% H₂O₂ + 0.1M, pH 4.0). The reaction was quenched with 1M phosphoric acid stop solution and OD measurements read at 450nm. OD values were calculated using values that fell within the linear portion of the standard curve and averaged for each sample. Urine creatinine was measured using the Jaffe assay [23]. Briefly, creatinine standard (Sigma-Aldrich, St. Louis, MO) and urine sample were incubated for 10 min at room temperature with distilled water, 0.75M NaOH and 1% picric acid and optical density read at 492nm.

Serum IgG ELISA

Total IgG levels in mouse sera were measured by ELISA as described previously [24]. Briefly, wells were pre-coated overnight with goat anti-mouse IgG (Southern Biotech, Birmingham, AL; cat#1030–01) in 0.05M Carbonate Bicarbonate buffer. Blocking and sample dilutions were performed with 1% BSA in PBS-Tween20 (BSA/PBST). Mouse IgG (Sigma-Aldrich cat# I-8765; St. Louis, MO) served as the standard and diluted in BSA/PBST. IgG was detected with HRP-conjugated goat-anti-mouse IgG (Southern Biotech, Birmingham, AL; cat# 1030–05) diluted in BSA/PBST. Wells were then incubated with TMB, reaction was quenched with 1M phosphoric acid stop solution, and OD measurements read at 450nm. OD values were calculated using values that fell within the linear portion of the standard curve and averaged for each sample.

Flow cytometry

Approximately one-quarter of each spleen was dissociated in RPMI 1640 as described previously [24] to generate single-cell suspensions. Red blood cells were lysed in ACK lysis buffer (Lonza, Walkersville, MD). Cells were suspended in flow buffer (PBS, 1% fetal bovine serum, 0.1% sodium azide) and Fc receptors blocked with anti-mouse CD16/CD32 (BD Bioscience, San Jose, CA). Live-dead fixable aqua dead cell stain (Thermo-Fisher, Waltham, MA) was used according to manufacturer's instructions to gate on live cells. The percentage of T cells expressing markers of activation were measured using a BD LSR Fortessa flow cytometer after staining with anti-CD3-FITC, CD44-APC, and CD62L-PE antibodies (clones: 145-2c11, IM7, and MEL-14 respectively, BD Biosciences, San Jose, CA). Live cells were gated on CD3 and then analyzed for CD44 and CD62L staining.

Renal pathology assessment

Fixed kidney sections from each of the study mice were stained with H&E or PAS and evaluated by a pathologist blinded to the treatment. Renal pathology scores were based on glomerular inflammation, proliferation, crescent formation, necrosis, and interstitial inflammation on a scale of 0 to 3 for each feature. Scores for each feature were totaled and presented in figures. Any observed crescent formation and necrosis were given double scores due to severity of those lesions.

Culture and stimulation of mesangial cells

Primary mesangial cells (MCs) were generated from glomeruli isolated from six week-old MRL/lpr female mice, characterized/phenotyped, and cultured as described previously [14]. MCs were stimulated with 10% serum collected and pooled from 16–18 week-old MRL/lpr mice (referred to as lupus serum). For OP treatments, MCs were incubated with 500 μ M OP 16–18 hrs prior to addition of lupus serum for 3 hrs. Significant production of IL-6 after addition of lupus serum was used to confirm stimulation in each experiment as demonstrated previously [14]. NEU activity and lipids were measured as described above.

Statistical analyses

All analyses were conducted in SAS v. 9.4 (SAS Institute, Cary, NC). In the MRL/lpr OP treatment study, associations between animal type (MpJ, lpr OP-treated, and lpr water-treated) with fold change in renal GM3, SMs, sphingolipids, Ceramide, HexCers, and LacCers for chain lengths C16, C22, C24, C24_1 and Total of all chain lengths (C14-C26) were evaluated using an ANOVA approach. Pairwise differences between animal types were evaluated using

linear contrasts with a Bonferroni adjustment. The associations between animal type with renal score, overall change in urine albumin, spleen weight, spleen:body weight ratio, and activated T cells were examined using an ANOVA or Kruskal-Wallis approach where appropriate. The association between animal type with urine albumin and NEU activity over time were evaluated using a linear mixed model (LMM) approach. LMMs included fixed effects for animal type, time, and the interaction between type and time and a random mouse/sample effect to account for correlation between repeated measures in the same mouse/sample over time. Pairwise differences between animal types were evaluated using linear contrasts with a Bonferroni adjustment.

In the MRL/lpr Genz-667161 treatment study, dose of Genz-667161 was considered both as continuous, to determine the potential dose response relationship, and categorical to examine mean response in each dose group. The association between continuous dose with lipid profiles and with spleen weight outcomes was evaluated using Pearson's or Spearman's rank correlation as appropriate. The associations between treatment dose with lipid profile and spleen weight outcomes were examined using an ANOVA approach. Pairwise differences between treatment groups were evaluated using linear contrasts. No adjustment for multiple comparisons was made due to the exploratory nature of this experiment.

For the MC culture experiment, association between treatment of lupus serum in the absence or presence of OP with GSL levels were evaluated using a linear mixed model approach. Models with one treatment variable included a fixed effect for treatment. All experiments were performed twice with similar results, data were combined for the statistical analyses, and representative graphs are presented. Model assumptions were checked graphically and transformations were considered as needed. Pairwise differences between groups were evaluated using linear contrasts and a Bonferroni correction was applied to adjust for multiple comparisons. Adjusted p-values are presented on the graphs.

Results

Effects of NEU inhibition on renal disease in MRL/lpr mice

To test if pharmacologically reducing neuraminidase (NEU) activity, which is elevated in MRL/lpr mice [13], would normalize GSL metabolism and improve disease, female MRL/lpr mice received 10 mg/kg of the NEU inhibitor oseltamivir phosphate (OP) or water daily for four weeks. Ten age-matched untreated MRL/MpJ mice were used as non-nephritic controls. Effects of OP treatment on kidney disease were analyzed by measuring proteinuria and assessing renal pathology. Albuminuria levels on average were similar between the water- and OP-treated MRL/lpr (lpr) mice prior to beginning treatment. Both lpr groups had significantly higher albuminuria than age-matched MRL/MpJ (MpJ) controls prior to beginning treatment and at the endpoint (Fig 1A). While most mice in both of the lpr groups had increases and a few mice had decreases in proteinuria over the treatment period (Fig 1A), on average, there was not a significant difference in the change in urine albumin between the OP- and water-treated groups (Fig 1B). There also was no significant difference in total renal pathology scores between the water- and OP-treated lpr groups, and both lpr groups had significantly higher renal pathology scores than the MpJ group (Fig 1C). Examples of pathological features observed in both the lpr water-treated (crescents) and OP-treated (hypercellularity) mice are shown compared to normal pathology in the MRL/MpJ mice (Fig 1D).

Effects of NEU inhibition on systemic disease in MRL/lpr mice

We previously demonstrated Neu1 levels and LacCers levels are elevated in MRL/lpr T cells from nephritic mice compared to age-matched non-nephritic mice and parallels their

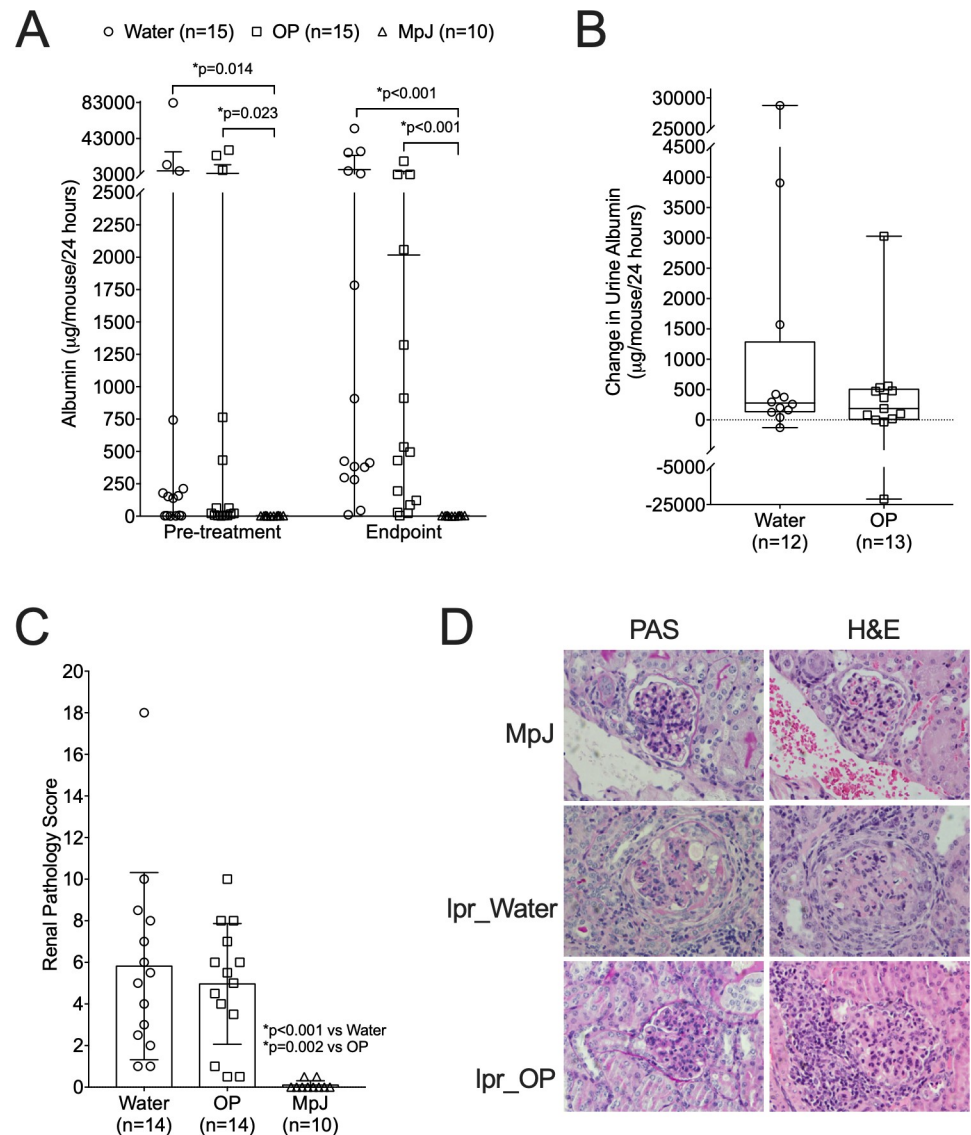


Fig 1. Renal disease measures are not significantly impacted by OP treatment in MRL/lpr mice. MRL/lpr female mice were treated with vehicle (Water) or oseltamivir phosphate (OP) at 12–13 weeks of age for 4 weeks. Untreated age-matched MRL/MpJ (MpJ) female mice served as non-nephritic lupus controls. Urine albumin was measured in 24 hr urine samples one day prior to beginning treatment (Pre-treatment) and one day prior to euthanasia (Endpoint). Both the absolute levels (A) and the change from Pre-treatment to Endpoint (B) in urine albumin are presented. (C) Renal pathology scores at the endpoint of the study. Means with standard deviations are presented. (D) Renal sections stained with H&E or PAS from the MRL/MpJ (MpJ) and MRL/lpr water-treated (lpr_Water) and OP-treated (lpr_OP) mice.

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hyperactivation *ex vivo* [24]. Therefore, we investigated if OP treatment impacted splenomegaly, the percentage of activated T cells, and serum IgG levels. As expected, the water-treated lpr mice had significantly larger spleens than the MpJ mice (Fig 2A). However, there were no differences in spleen size (Fig 2A) or spleen to body weight ratio (Fig 2B) and no differences in the percentage of activated T cells (Fig 2C) between the OP-treated and water-treated MRL/lpr groups. Serum IgG levels were significantly higher in the water-treated lpr mice compared to the MpJ mice just prior to treatment and at the study endpoint and both groups showed significant increases over the four weeks of the study (Fig 2D). Although not significant, there was

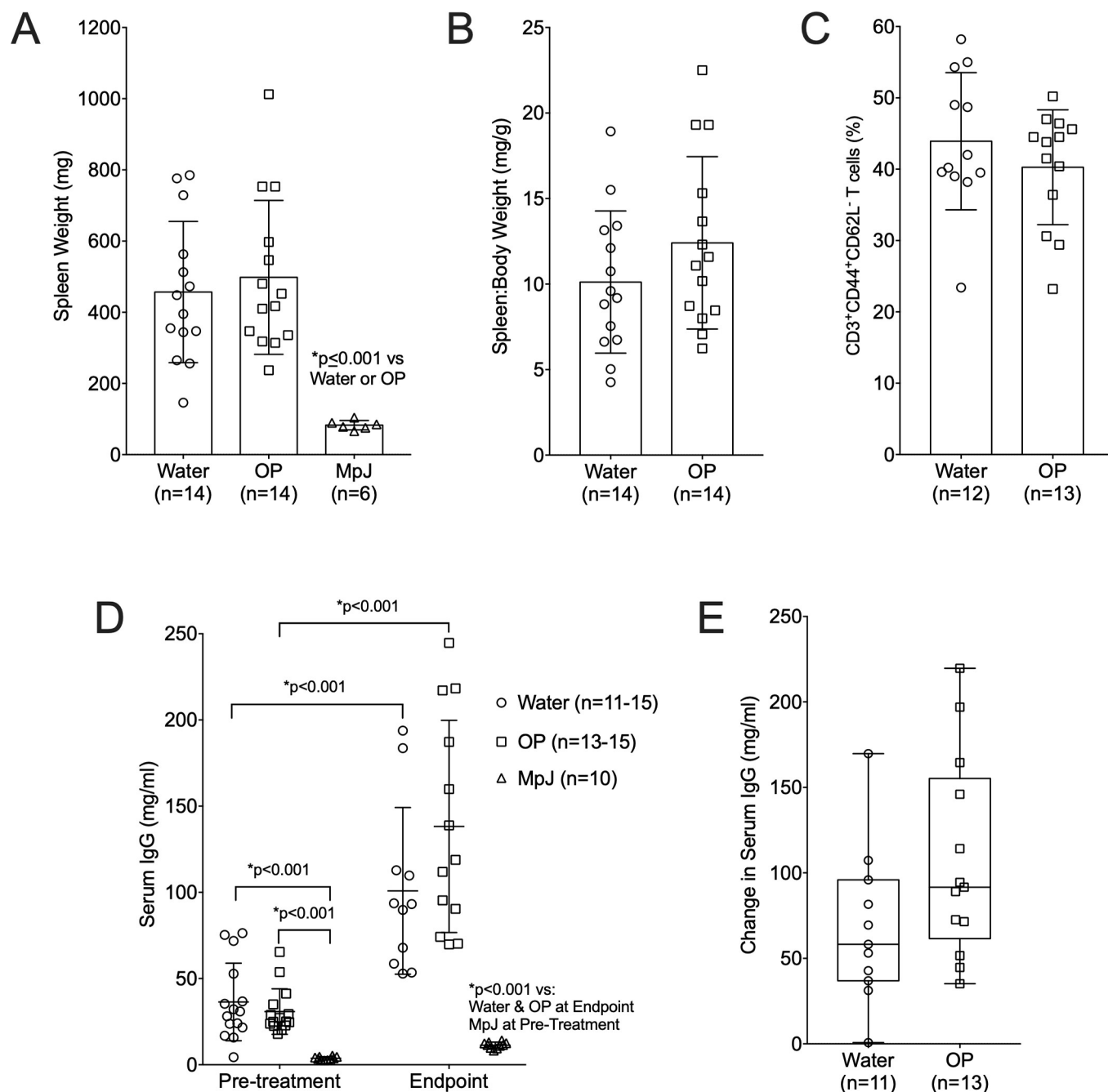


Fig 2. Systemic disease measures are not significantly impacted by OP treatment in MRL/lpr mice. Splenomegaly in the same mice as in Fig 1 was determined by absolute spleen weight (A) and as spleen to body weight ratio (B). (C) Percentages of activated/memory T cells (CD3⁺ CD44⁺ CD62L⁻) were measured in spleen by flow cytometry. IgG levels were measured by ELISA in serum samples collected one day prior to treatment (Pre-treatment) and one day prior to euthanasia (Endpoint) with absolute levels (D) and change in levels from Pre-treatment to Endpoint (E) presented. Means with standard deviations are presented. Water, water-treated MRL/lpr mice; OP, OP-treated MRL/lpr mice; MpJ, MRL/MpJ (untreated) mice.

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an unexpected slight increase in the absolute levels of serum IgG at the endpoint (Fig 2D) and in the change in levels from pre-treatment to endpoint (Fig 2E) in the OP-treated compared to the water-treated lpr groups.

Effects of NEU inhibition on renal GSL metabolism in MRL/lpr mice

We previously demonstrated significantly elevated levels of renal LacCers, GlcCers, and NEU activity in nephritic MRL/lpr (lpr) mice compared to age-matched non-nephritic MRL/MpJ (MpJ) lupus mice and healthy C57BL/6 controls, but the levels of ceramide and the message levels of synthesis pathway enzymes were not significantly elevated [13]. These results suggested NEU-mediated catabolism of gangliosides may be responsible for the elevated LacCer or GlcCer levels in the kidney. Therefore, we compared the levels of renal ganglioside GM3, the simplest sialic acid-containing ganglioside (LacCer with a single sialic acid) and most abundant ganglioside [25], in the water-treated lpr and untreated MpJ mice at the endpoint. Surprisingly, all the major GM3 chain-length species except C24:1, as well as total (all chain lengths from C14 to C26:1) GM3 levels, were significantly elevated in the lpr compared to the MpJ mice (Fig 3A). We also measured the levels of lipids that feed into the GSL pathway through ceramide, including sphingomyelin (SM), sphingolipid-1 phosphate (S1P), sphingosine (So), dihydrosphingolipid-1 phosphate (DHS1P), and dihydrosphingosine (DHSO). Excluding C16, all of the SM chain lengths and total SM levels are significantly elevated (Fig 3B) as are S1P, So, DHS1P, DHSO (Fig 3C) in the kidney of the water-treated lpr mice compared to their age-matched non-nephritic MpJ counterparts. Fig 3D depicts the lipids and enzymes in the GSL pathway and the lipids that feed into the GSL pathway through Cer. Enzymes and lipids in bold text were demonstrated to be elevated in the kidneys of female nephritic lpr compared to female MpJ age-matched (non-nephritic) mice previously [13] and in this study.

Inhibiting NEU activity and blocking the catabolism of GM3 to LacCer was expected to result in accumulation of renal GM3 levels in the OP-treated mice. Although differences were not significant, the longer chain-length GM3 species (C24, C24:1) and total GM3 levels showed a trend towards being increased in the kidneys of OP-treated mice compared to the water-treated lpr mice. This was supported by the smaller p values for OP-treated lpr versus MpJ compared to those for the water-treated lpr versus MpJ for C22, C24, and total GM3. The OP-treated lpr mice also showed a trend towards slight increases in renal SM (Fig 3B), but not for S1P, So, DHS1P, and DHSO levels (Fig 3C) compared to water-treated lpr mice. The elevated renal levels of SMs, S1P, So, DHS1P, and DHSO in MRL/lpr mice with nephritis compared to the non-nephritic lupus MRL/MpJ mice suggest GSLs may be elevated due in part to increased synthesis through ceramide (Fig 3D).

Previously, we observed most of the renal hexosylceramides (HexCers) chain lengths, composed of GlcCers and galactosylceramides (GalCers), were significantly elevated in lpr compared to MpJ female mice [13]. We further demonstrated the increased levels of HexCers were due to increased GlcCers. Therefore, due to ease and cost, we measured HexCer levels and consider them representative of GlcCers. Similar to our previous results, we observed a significant increase in renal C24 Cer in the water-treated lpr compared to the MpJ mice (Fig 4A). However, we observed renal C22, C24:1, and total Cers were also significantly elevated in the water-treated lpr compared to the MpJ mice. Elevated levels of C16, C22, C24, and total HexCers (Fig 4B) and C16, C24:1, and total LacCers (Fig 4C) were all elevated in the water-treated lpr compared to the MpJ mice as observed previously [13]. The overall levels of renal HexCers and LacCers were expected to decrease in the OP-treated mice due to decreased NEU-mediated catabolism of gangliosides to LacCer (and subsequent catabolism to GlcCer). However, Cers levels were not significantly different (Fig 4A) and there was a trend towards increased levels of renal HexCers (Fig 4B) and LacCers (Fig 4C) in the OP-treated compared to the water-treated lpr mice.

To determine if the OP treatment impacted renal NEU activity as expected, we measured renal NEU activity. Both the water-treated and OP-treated lpr mice had a significant increase

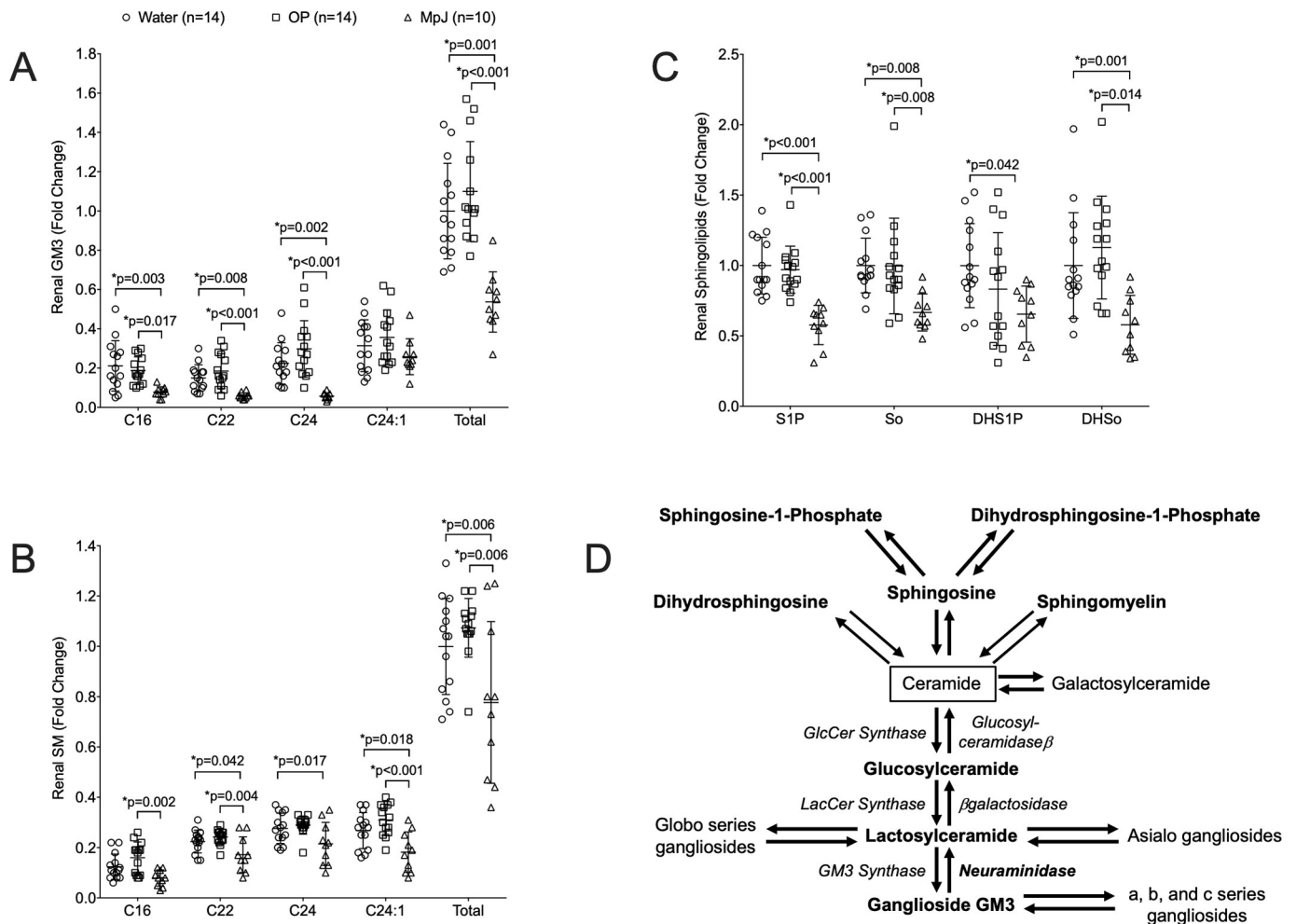


Fig 3. Elevated renal GM3s and Sphingolipids in nephritic MRL/lpr are not significantly impacted by treatment with OP. Renal lipids were quantified in renal cortex homogenates from the same mice as in Fig 1 at the endpoint of the study. Major chain length species (C16, C22, C24, C24:1) and total of all chain lengths (Total) are presented for each animal for: (A) Ganglioside GM3s and (B) Sphingomyelins (SMs). Overall levels are presented for each animal for: (C) Sphingosine-1 phosphate (S1P), sphingosine (So), dihydrosphingosine-1 phosphate (DHS1P), and dihydrosphingosine (DHSO). Means with standard deviations are provided on all graphs. Water, water-treated MRL/lpr mice; OP, OP-treated MRL/lpr mice; MpJ, MRL/MpJ (untreated) mice. (D) Schematic of GSL metabolism and the pathways feeding into it. Enzymes are in italics. Lipids and enzymes in bold are elevated in kidneys of nephritic female MRL/lpr compared to age-matched female MRL/MpJ mice.

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in NEU activity compared to MpJ mice (Fig 4D, $p < 0.001$) as expected based on previous observations [13]. However, NEU activity levels were not significantly different between the water- and OP-treated lpr mice. GSL levels and NEU activity in the kidney could only be measured at the endpoint of the study. Since the kidneys were collected 24 hrs after the last dose of OP, 12–14 hrs beyond the half-life of the drug and NEU activity may have recovered by the time of organ collection, we measured lipid levels in the urine samples of the first set of MRL/lpr water- and OP-treated mice. Urine was collected over 24 hrs and may be a better indicator if OP impacted GSL metabolism as it would capture the period during highest drug activity. Urine levels of most HexCers and LacCers in the endpoint urine samples were higher in the OP-treated mice compared to the water-treated mice, and significantly higher when totaling all chain lengths; HexCers (Fig 4E, $p < 0.0001$) and LacCers (Fig 4F, $p = 0.0036$). Similar increases were observed in the Cers (S1A Fig) and SMs (S1B Fig) in the endpoint urine

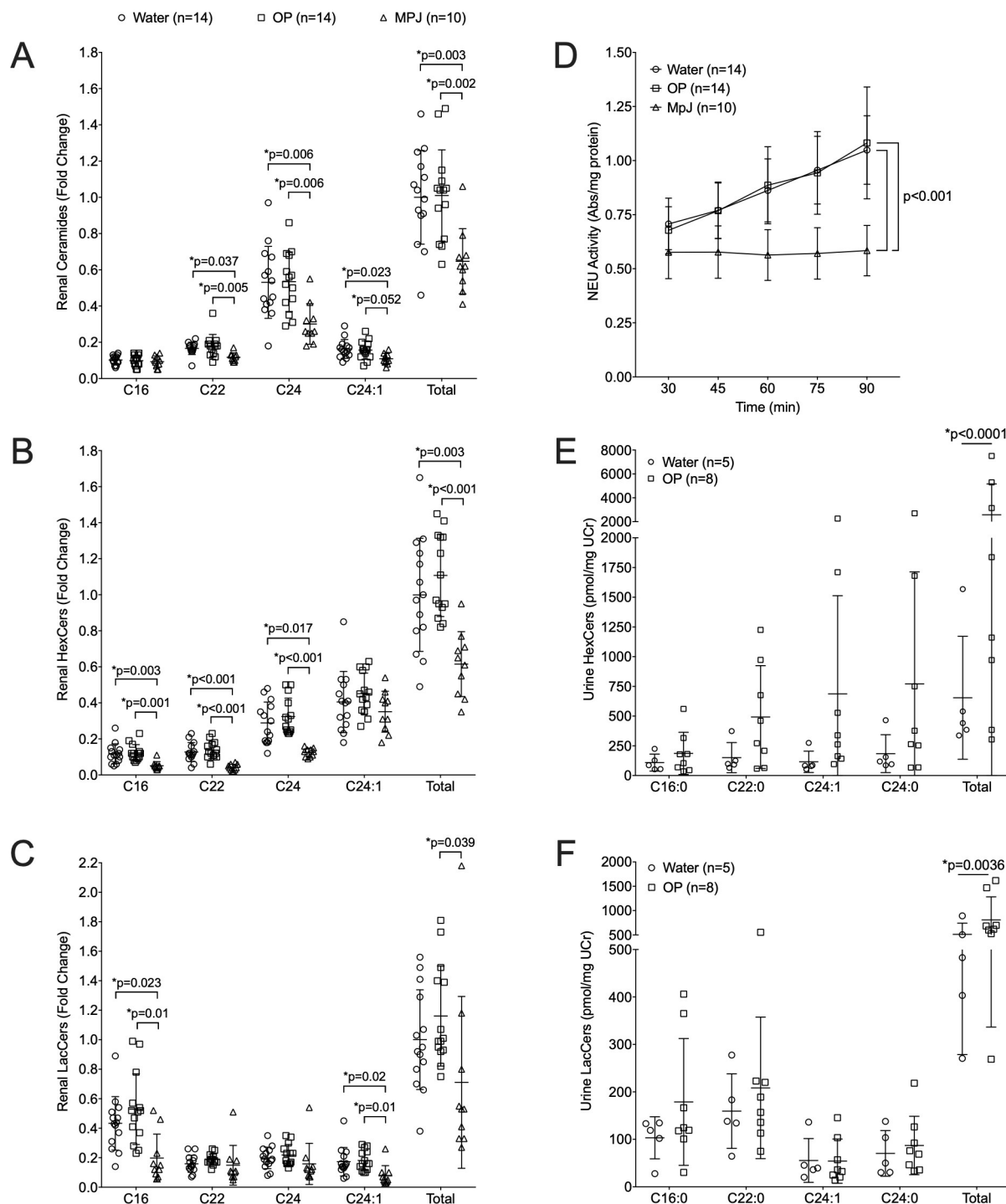


Fig 4. Renal and urine HexCers and LacCers are increased in OP-treated MRL/lpr mice. Renal GSLs were quantified in renal cortex homogenates from the same mice as in Fig 1 at the endpoint of the study. (A) Ceramides (Cers). (B) Hexosylceramides (HexCers). (C) Lactosylceramides (LacCers). Major chain length species (C16, C22, C24, C24:1) and total of all chain-lengths (Total) of each lipid presented for each animal are presented in the graphs. (D) Neuraminidase (NEU) activity was quantified in kidney cortex homogenates. NEU activity (absorbance, Abs) was quantified within each homogenate at 15 min intervals, normalized to total protein, and presented as average absorbance per mg protein for each time point. HexCers (E) and LacCers (F) were measured in 24-hr urine collections at the endpoint of the study and normalized to urine creatinine (UCr). Means with standard deviations are provided for all graphs. Water, water-treated MRL/lpr mice; OP, OP-treated MRL/lpr mice; MpJ, MRL/MpJ (untreated) mice.

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samples from OP-treated mice with significant increases observed in total Cers ($p = 0.0006$), and in C16 ($p = 0.0012$) and total ($p < 0.0001$) SMs compared to water-treated mice.

To confirm the observed increases in urine lipids at the endpoint were not due to differences in the starting baseline (pre-treatment) levels, the change in urine lipids from baseline to endpoint samples were calculated. These results confirm urine total HexCers (S1C Fig), LacCers (S1D Fig), Cers (S1E Fig), and SMs (S1F Fig) significantly increased in OP-treated compared to water-treated mice. Together, the trend towards increased renal GSLs and significant increases in urine GSLs at the endpoint of the study suggest OP treatment increased rather than decreased GSL levels. To determine if OP treatment can increase GSLs, we stimulated primary mesangial cells from pre-disease MRL/lpr mice in the absence or presence of OP. OP significantly decreased NEU activity (Fig 5A) and increased HexCers, LacCers, and GM3s (Fig 5B), as well as Cers, So, and DHSO in stimulated lupus prone mesangial cells (Fig 5C). These *in vitro* results suggest blocking GSL catabolism by inhibiting NEU activity (with OP) may result in accumulation of GSLs in lupus prone mice.

Inhibiting GlcCer synthase decreased renal GSL levels in MRL/lpr mice

Results from the OP study suggest inhibiting NEU-mediated GSL catabolism may increase rather than decrease already elevated GSL levels, which may exacerbate disease. Therefore, we performed a pilot study to determine if inhibiting the GSL synthesis pathway will reduce GSLs levels. For this study, female MRL/lpr mice were treated with the experimental, proprietary GlcCer synthase inhibitor Genz-667161, which was used to treat mouse models of Gaucher disease and amyotrophic lateral sclerosis [26, 27]. MRL/lpr mice were treated with Genz-667161 at 0, 1, 3, 10, or 20 mg/kg/day. Treatment was started at 12–13 weeks of age for four weeks. Twenty-four-hour urine collections were obtained just prior to treatment and weekly until the end of the study. Serum was collected at the end of the study.

There was a significant negative correlation between dose with HexCer levels for all chain lengths ($p < 0.001$ for all) (Fig 6A). Pairwise differences between Genz-667161 doses were also evaluated. Mean differences between each dose and untreated (0 mg) by chain length for each lipid were compared and p-values for the pairwise comparisons are provided on the graphs for each GSL. The most notable pairwise effects were on HexCers levels. All doses ≥ 3 mg showed significant decreases for all chain lengths of HexCer compared to untreated (0 mg) mice (Fig 6A, p-values indicated on graph). Moreover, the 20 mg dose also resulted in significant decreases for C22 ($p = 0.006$), C24:1 ($p = 0.018$), and total ($p = 0.016$) HexCers relative to the 3 mg dose (Fig 6A). Genz-667161 blocks synthesis of Cers to GlcCers; therefore, the decrease in HexCers is due to decreased GlcCers and not GalCers. There were also significant effects observed on LacCer and GM3 levels. Dose was negatively correlated with C22 and total LacCers levels ($p < 0.05$) (Fig 6B) and with all GM3 chain lengths at $p < 0.01$ except C24:1, which was at $p < 0.05$ (Fig 6C). When pairwise comparisons among doses were evaluated, decreases for each LacCer chain length did not reach significance (Fig 6B) and only C16 GM3 at 10mg and 20mg doses compared to 0 mg reached significance (Fig 6C, p-values indicated on graph). There were no significant differences in Cers C16, C22, C24, C24:1, or total Cer with any dose (Fig 6D).

Based on the significant decreases in renal GSL levels, the effects of Genz-667161 on renal disease and systemic disease were evaluated. The MRL/lpr strain shows significant variability in onset and extent of disease expression with respect to nephritis. Since the three mice in the untreated group all showed low levels of urine albumin, effects of drug treatment on urine albumin was inconclusive (S2 Fig). Renal pathology score (Fig 6E) was not significantly associated with dose of Genz-667161 (treating dose as either continuous- $p = 0.112$, or categorical-

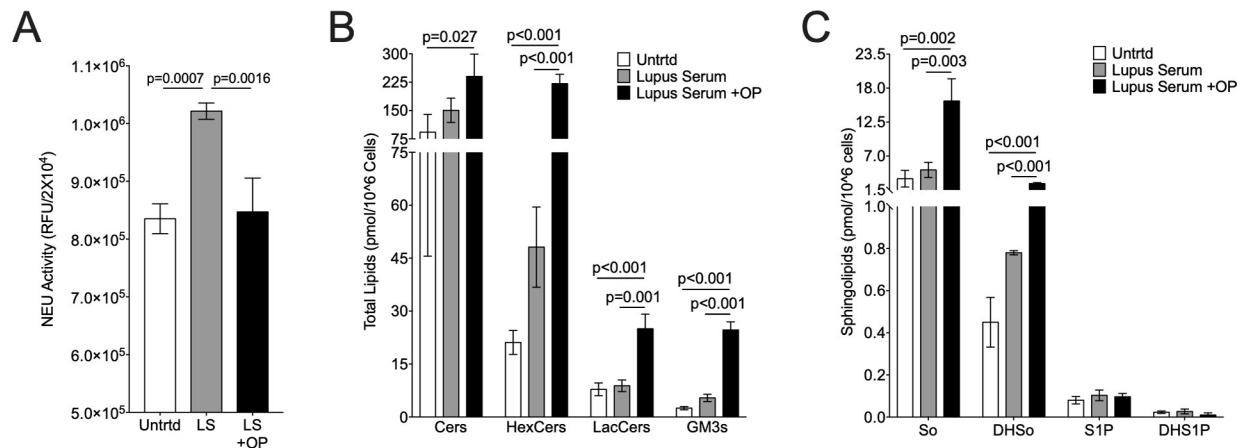


Fig 5. GSLs are significantly increased in OP-treated primary mesangial cells. Primary MRL/lpr mesangial cells were incubated with OP or vehicle (water) prior to addition of 10% MRL/lpr lupus serum or left untreated. A) NEU activity was measured in live cells. B) Levels of Cers and glycosphingolipids HexCers, LacCers, and GM3s were quantified for each chain length and totaled. C) Sphingolipids sphingosine (So), dihydrosphingosine (DHSO), sphingosine-1 phosphate (S1P), and dihydrosphingosine-1 phosphate (DHS1P) were quantified. Lipid levels are presented as pmol lipid per 10⁶ cells. Means with standard deviations are provided for all graphs. P-values were calculated as described in the materials and methods.

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$p = 0.219$). However, spleen to body weight ratio was negatively correlated (Fig 6F, $\rho = -0.535$, $p = 0.040$) and serum IgG levels were marginally correlated (Fig 6G, $\rho = -0.493$, $p = 0.062$) with increasing dose. These results suggest inhibiting the GSL synthesis pathway may provide a therapeutic effect in female lupus mice.

Discussion

Glycosphingolipids are neutral lipids that play important roles in cellular signaling in many cell types. GSLs are enriched in the kidney and GSL metabolism is altered in several kidney diseases, including lupus nephritis [6, 7, 10, 13]. GSL metabolism also was shown to be altered in T cells from patients with lupus [28–31] and in lupus mice [24, 32]. Specifically, we demonstrated that in lupus mice, LacCers, GlcCers, and NEU activity levels were increased in the kidney of mice with worse proteinuria [13, 14] and decreased in T cells of lupus mice with improved disease [24]. Although these results suggest the increased LacCer and GlcCer levels in the kidney were likely a result of increased catabolism of gangliosides due to increased NEU activity, ganglioside levels were not previously analyzed. In this study, we demonstrated that, despite increased NEU activity, renal GM3 levels were actually higher in kidneys of nephritic MRL/lpr mice compared to non-nephritic MRL/MpJ mice. Moreover, we demonstrated SMs, S1P, So, DHS1P, and DHSO are also elevated in the kidneys of nephritic MRL/lpr mice compared to the kidneys of non-nephritic MRL/MpJ mice. Our previous results [13] and the results presented here suggest the upregulation of the GSL pathway in the LN kidney may be due in large part to a general increase in lipids that feed into the GSL pathway through Cer, which we observed were elevated for many more Cers chain lengths in this set of mice in contrast to our previous results [13]. Determining the mechanisms by which the renal GSLs accumulate will likely provide critical insight into the pathophysiology of LN. Since the message level of GM3 synthase was observed to be similar while NEU activity was increased [13], the higher GM3 levels in the MRL/lpr kidney could be due to increased GM3 synthase activity and/or increased catabolism of more complex sialic acid-containing gangliosides to GM3.

Four mammalian NEUs exhibiting different tissue specificities have been identified. NEU1 is the major NEU expressed in the kidney with Neu1 message levels at approximately 10-

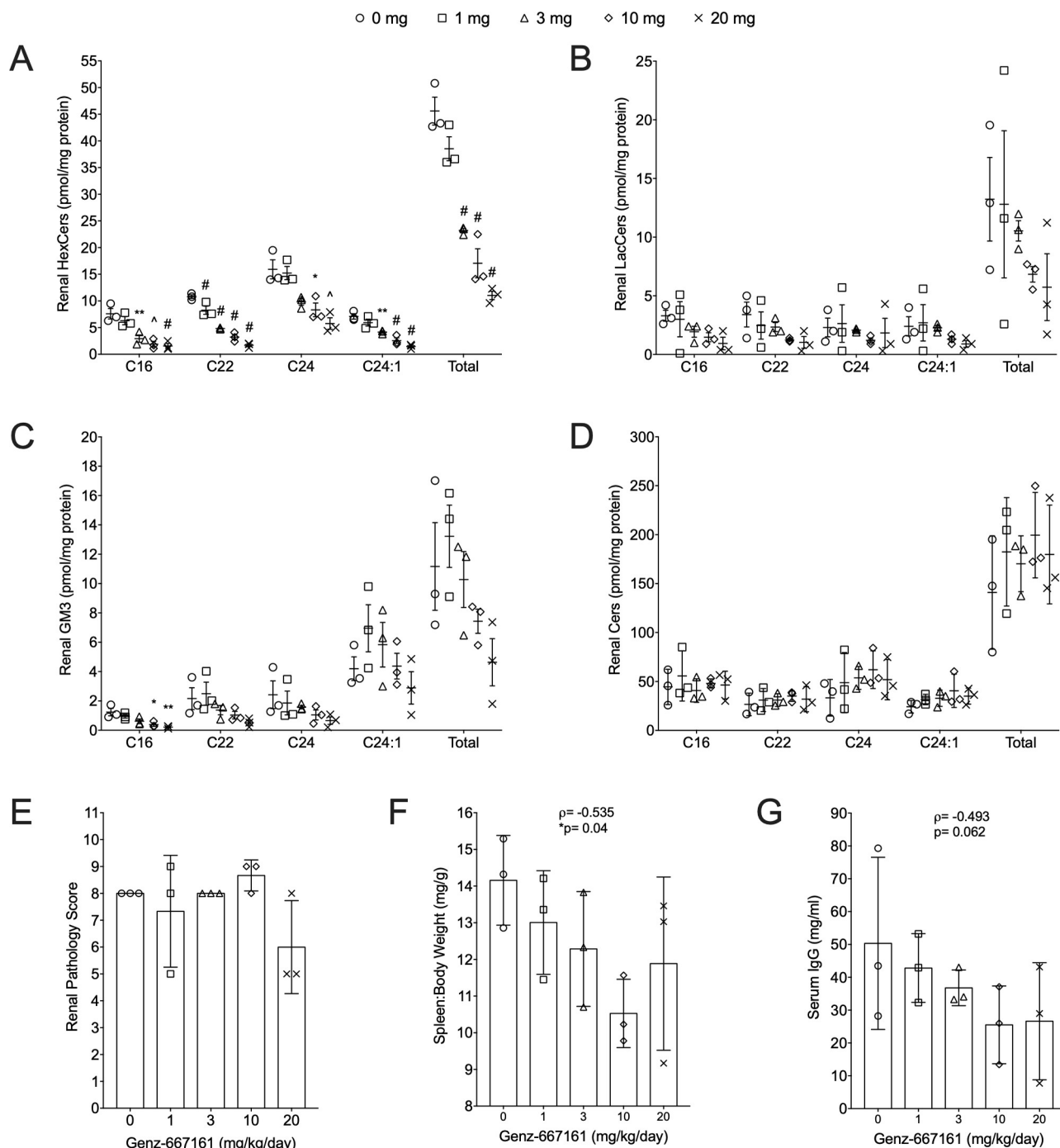


Fig 6. Decreased renal GSLs, splenomegaly, and serum IgG in MRL/lpr mice treated with a GlcCer synthase inhibitor. Female MRL/lpr mice were treated with 0, 1, 3, 10, or 20 mg/kg/day of the GlcCer synthase inhibitor Genz-667161 ($n = 3$ for each treatment group). Lipids were quantified in renal cortex homogenates at the endpoint of the study. All chain lengths of renal HexCers (A), LacCers (B), GM3s (C), and Cers (D) were quantified. The major chain length species and the total of all chain lengths (Total) are presented for each animal with means and standard errors indicated. There is a significant negative correlation between dose and: HexCer ($p < 0.001$ for all chain lengths); GM3 ($p < 0.01$ for C16, C22, C24, Total and $p < 0.05$ for C24:1); and LacCer ($p < 0.05$ for C22 and Total). P-values for pairwise comparisons between each treatment group with untreated (0 mg) are presented on the graphs: * $p \leq 0.05$, ** $p \leq 0.01$, ^ $p \leq 0.005$, # $p \leq 0.001$. Renal pathology scores (E), Ratio of spleen weight to body weight (F), and Serum IgG levels (G) were measured at the endpoint of the study. P-values for correlations between dose and outcome in E-G are provided on the graphs.

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50-fold higher than Neu3 in human and mouse while Neu2 and Neu4 are expressed at low to undetectable levels in the kidney [14, 33–35]. NEU1 and NEU3 protein expression within the kidney has not been analyzed in detail; however, we demonstrated NEU1 and NEU3 expression in the glomeruli of mouse renal sections appear to be higher in lupus mice with nephritis compared to lupus mice without nephritis [14]. Primary mesangial cells (MCs) from kidneys of MRL/lpr mice upregulated NEU activity and knocking down Neu1 message levels or blocking NEU activity significantly decreased IL-6 secretion upon stimulation *in vitro* [14]. Together, these results suggested accumulation of renal HexCers and LacCers in lupus mice [13, 14] may be due to increased break down of gangliosides by NEUs. Based on these observations we hypothesized that blocking NEU activity may normalize GSLs levels and improve disease. However, our results here blocking NEU activity and the catabolic pathway using the NEU inhibitor OP in MRL/lpr mice resulted in a trend towards increased renal and significantly increased urine GSL levels. Blocking NEU activity with OP in primary lupus serum-stimulated MCs *in vitro* also resulted in a significant increase in GSLs.

As noted above, MRL/lpr kidneys accumulate HexCers, LacCers, and NEU activity increases as disease progresses [13], which suggested increased catabolism may be responsible for the accumulation of GSLs. Our results here demonstrate that GM3 also accumulates during disease despite the increase in NEU activity. If GSL accumulation in MRL/lpr kidneys is due to upregulation of the synthesis pathway, blocking the catabolic pathway could potentially cause further accumulation of renal GSLs (see Fig 3D). We speculate the observed increase in renal NEU activity as disease progresses we reported previously [13, 14] may be a response to elevated GSL metabolism and reduce GM3s accumulation in the kidney. Interestingly, a complete knockout of NEU1 in the C57BL/6 strain resulted in pathological effects similar to those observed in lupus, including splenomegaly and proteinuria [36]. Hence, if increased NEU activity is a protective response for removing accumulating GM3s (and other gangliosides) in LN, blocking NEU may actually exacerbate disease. Thus, the trends of increasing renal GSLs in response to treatment with OP may be specific to lupus or specifically the MRL/lpr strain.

It is important to note that there are several potential limitations to our study that also could account for the lack of significant effects on the renal levels of GSLs or disease measures. First, OP was developed as a competitive inhibitor of viral NEU and is not as effective against mammalian NEUs. Several studies demonstrated OP blocks endogenous mammalian NEU activity *in vitro* and *in vivo* with concentrations in the μM range depending on the cell type and study (compared to nM range for viral NEUs) [20, 37–43]. We chose a 10 mg/kg dose based on previous studies in mice demonstrating it was effective in blocking mammalian neuraminidase activity *in vivo* to decrease GM1 levels in T cells [20] and reduce bleomycin-induced lung fibrosis [21]. Although many of these studies suggest OP inhibits NEU1, the major NEU expressed in the kidney, activity of the other NEUs could be compensating if OP does not effectively inhibit all four mammalian NEUs. Second, OP is a prodrug for the active metabolite oseltamivir carboxylate (OC), a structural homolog of sialic acid, and the plasma half-life of OC is approximately 6–10 hours after oral administration. Although a 10 mg/kg/day OP dose in mice was shown to have similar plasma levels of OC as humans administered 75 mg/kg OP twice daily, [19], the once daily administration of OP in this study may not have sustained a steady-state reduction in NEU activity in the kidney, especially since we did not observe any difference in renal NEU activity at the end point of the study. Therefore, the dosage used may have been insufficient to block any, or all, NEU activity. Ideally, development of specific mammalian NEU inhibitors would more accurately determine how, or if, blocking all NEU activity or the activity of a specific NEU will impact renal GSL levels and disease measures in LN.

The unexpected elevated GM3 levels in the MRL/lpr mice compared to the non-nephritic MRL/MpJ parental strain and the results of the OP-treatment study suggest elevated GSL

metabolism may be due to continued or increased GSL synthesis rather than increased GSL catabolism. We therefore, tested different doses of a GlcCer synthase inhibitor developed by Genzyme to block the synthesis of GlcCer from Cer. Although this was a pilot study with only three mice per dosage group, the observed significant dose-responsive decrease in GSLs levels including HexCers and GM3s, and negative correlations of splenomegaly and serum IgG with increasing dose suggest strongly that targeting the GSL synthesis pathway may normalize renal GSL levels and improve disease. Another Genzyme GlcCer synthase inhibitor (Genz-123346) reduced renal GlcCer and GM3 levels and improved disease in a mouse model of PKD [7]. Thus, GSL metabolic dysfunction may be a common pathophysiologic pathway in LN and PKD. Based on our pilot study, a larger study with more mice and a longer treatment period is warranted to definitively determine if blocking the GSL synthesis pathway will significantly impact both renal disease and systemic disease.

Studies to identify the mechanisms mediating elevated GSL metabolism during disease progression are needed to better understand the underlying mediators of LN. We previously showed mRNA expression of the synthesis pathway enzymes are not significantly increased in MRL/lpr kidneys [13]. Therefore, upregulation of the synthesis pathway may be a result of increased activity of one or more synthesis enzymes or increased availability of Cers generated from accumulated SMs, So, or DHSO. Alternatively or additionally, infiltrating immune cells may be contributing to the observed elevated renal GSLs and/or NEU activity levels. These potential mechanisms are under investigation and remain to be determined. Defining the mechanisms by which renal GSL metabolism becomes elevated and the pathophysiological effects of elevated GSLs will provide a greater understanding of LN disease development and progression.

In conclusion, we demonstrated renal GM3s are elevated in LN mice despite increased levels of renal NEU activity that catabolizes GM3s. Additionally, we demonstrated SMs and several sphingolipids that feed into the GSL pathway through Cer are also elevated in LN mice. The OP and Genz-667161 treatment study results suggest blocking the GSL catabolic pathway may further elevate renal GSL levels while blocking the synthesis pathway may be effective in normalizing GSL metabolism in nephritic lupus mice. This may be translationally relevant to human patients with lupus nephritis who have elevated urine GSLs levels [13]. It is also possible that blocking both the synthesis and catabolic pathways may be necessary to effectively normalize renal GSL metabolism and attenuate disease expression/progression in lupus nephritis. Alternatively, upregulated GSLs may be playing a protective role, and normalizing renal GSL metabolism may exacerbate nephritis. Hence, further studies to understand the pathophysiologic impact of elevated GSL metabolism in lupus nephritis is needed.

Supporting information

S1 Fig. Urine Cers and SMs and change in urine lipids in OP-treated MRL/lpr mice. HexCers, LacCers, Cers, and SMs were measured in baseline and endpoint 24-hr urine collections and normalized to urine creatinine (UCr). Levels of Cers (A) and SMs (B) in endpoint urine samples. Difference from baseline to endpoint levels of HexCers (C), LacCers (D), Cers (E), and SMs (E). Major chain length species (C16, C22, C24, C24:1) and total of all chain-lengths (Total) of each lipid are presented for each animal and means with standard deviations are provided for all graphs. Water, water-treated MRL/lpr mice; OP, OP-treated MRL/lpr mice. (TIFF)

S2 Fig. Urine albumin levels in Genz-667161-treated mice. Urine albumin was measured in 24 hr urine samples one day prior to beginning treatment and one day prior to euthanasia. The change from Pre-treatment to Endpoint is presented. Means with standard deviations are

presented.
(TIFF)

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The role of neuraminidase in TLR4-MAPK signaling and the release of cytokines by lupus serum-stimulated mesangial cells

Running title: *NEU activity and TLR4-MAPK signaling in mesangial cells*

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List of Abbreviations: Mesangial Cells (MCs), Neuraminidase (NEU), Neuraminidase 1-4 (NEU1-NEU4), Systemic lupus erythematosus (SLE), Lupus Nephritis (LN), Glycosphingolipid (GSL), Lupus Serum (LS), C57BL/6 (B6), Oseltamivir Phosphate (OP), Lipopolysaccharide (LPS), Sialic Acids (SAs)

SUMMARY

Previously, we demonstrated neuraminidase (NEU) activity or NEU1 expression, specifically, are increased in the kidneys of lupus mice and urine of human patients with nephritis. Additionally, NEU activity mediates IL-6 secretion from lupus prone MRL/lpr primary mouse mesangial cells (MCs) in response to an IgG mimic. IL-6 mediates glomerular inflammation and promotes tissue damage in patients and mouse strains with lupus nephritis. This study further elucidates the mechanisms by which NEU activity and NEU1 specifically mediates the release of IL-6 and other cytokines from lupus prone MCs. We demonstrate significantly increased release of multiple cytokines and NEU activity in MRL/lpr MCs in response to serum from MRL/lpr mice (lupus serum). Inhibiting NEU activity significantly reduced secretion of three of those cytokines; IL-6, GM-CSF, and MIP1 α . Message levels of *Il-6* and *Gm-csf* were also increased in response to lupus serum and reduced when NEU activity was inhibited. Neutralizing antibodies to cell-surface receptors and MAPK inhibitors in lupus serum- or LPS-stimulated MCs indicate TLR4 and p38 or ERK MAP kinase signaling play key roles in the NEU-mediated secretion of IL-6. Significantly reduced IL-6 release was observed in C57BL/6 (B6) Neu1 $\pm$ primary MCs compared to wild-type (Neu1 $^{+/+}$) B6 MCs in response to lupus serum. Additional results show inhibiting NEU activity significantly increases sialic acid-containing N-glycan levels. Together, our novel observations support a role for NEU activity, and specifically NEU1, in mediating release of IL-6 from lupus prone MCs in response to lupus serum through a TLR4-p38/ERK MAPK that likely includes desialylation of glycoproteins.

INTRODUCTION

Systemic lupus erythematosus (SLE) is an inflammatory, chronic autoimmune disorder involving multiple organs. Defects in the clearance of apoptotic cells lead to intracellular autoantigen release, autoantibody production, and immune complex deposition and inflammation in target organs. Glomerulonephritis is the leading cause of morbidity and mortality in lupus patients; however, the mediators of lupus nephritis (LN) remain incompletely known. Glycosphingolipid (GSL) metabolism is altered in lupus patients and lupus prone

mice with nephritis compared to their non-nephritis counterparts and healthy controls ¹, sharing similarities with other chronic kidney diseases shown to be mediated by GSL metabolism ²⁻⁵. However, the precise mechanisms by which GSL metabolism impacts chronic kidney diseases, including lupus nephritis, have not been elucidated. Proinflammatory cytokines are elevated in the kidney of mice and patients with lupus. IL-6 in particular plays a key role in the onset of nephritis in lupus mice ⁶⁻¹¹. Altered GSL metabolism in MRL/lpr kidneys included increased activity of neuraminidase ¹, which we demonstrated mediates IL-6 release from primary MRL/lpr mesangial cells (MCs) stimulated with an IgG mimic or serum from MRL/lpr mice *in vitro* ¹².

Four mammalian neuraminidases (NEUs) (or sialidases) remove sialic acids from glycolipids and glycoproteins. NEU1 is typically located in the lysosome and translocated to the plasma membrane of many cell types upon activation ¹³⁻¹⁶. NEU2 and NEU3 are located in the cytosol and plasma membrane, respectively ^{17, 18}, while NEU4 is found in the mitochondria and lysosome ^{19, 20}. NEUs also differ somewhat in substrate specificities and tissue distribution. NEU1 is ubiquitously expressed and is the major NEU expressed in the kidney ^{17, 19, 21-24}. The only other NEU detectable in the kidney is NEU3 with message levels of Neu1 expressed approximately 40-fold higher than Neu3 ¹⁹ and NEU1 and NEU3 proteins are readily detected in the glomeruli of renal tissue sections of MRL/lpr lupus mice ¹². While NEU1 was largely observed to desialylate glycoproteins, NEU3 showed a preference for glycolipids (gangliosides) ²⁵⁻²⁹ and their activity at the plasma membrane impacts receptor activation and signaling in a variety of cell types ²⁹⁻³⁵.

To extend our previous studies and further elucidate the mechanisms by which NEU activity mediates MC function, we investigated the potential signaling pathways involved in NEU-mediated cytokine release in response to lupus serum. The activation of lupus prone MCs by soluble mediators present in lupus serum is not well established. Using lupus serum as a disease-relevant stimulator, we determined NEU activity mediates the release of GM-CSF and MIP1 α in addition to IL-6 by MRL/lpr lupus prone primary MCs. NEU activity likely mediates IL-6 release directly while effects on GM-CSF and MIP1 α release are likely indirectly mediated by NEU activity. NEU-mediated release of IL-6 occurs in part through TLR4-MAPK p38 or ERK signaling pathways in response to lupus serum. We also provide evidence supporting NEU-mediated cytokine release by MCs may involve removal of sialic acid residues from specific N-glycans.

MATERIALS AND METHODS

Reagents

Antibodies used include anti-FcγR3 (anti-CD16, rat anti-mouse #MAB19601) and anti-TNFR1/TNFRSF1A (hamster anti-mouse #MAB430100) from R&D Systems (Minneapolis, MN); anti-FcγR2/3 (anti-CD16/CD32, rat anti-mouse #101301), anti-FcγR4 (anti-CD16.2, hamster anti-mouse #149502), anti-TLR2 (rat or mouse anti-mouse, #148601 or #121801), and anti-TLR4/MD2 (rat anti-mouse, #117607) from BioLegend (San Diego, CA); anti-TNFR2 (hamster anti-mouse #BE0247) from BioXCell (West Lebanon, NH); anti-Annexin A2 (Abcam, Cambridge, MA); isotype controls hamster IgG (#400901) and mouse IgG (#400101), and rat IgG (#400501) from BioLegend; phospho-p38 MAPK (Thr180/Tyr182, rabbit anti-mouse/human, #9211), p38 MAPK (rabbit anti-mouse/human, #9212), phospho-p44/42 ERK1/2 MAPK (Thr202/Tyr204, rabbit anti-mouse/human, #9101), and p44/42 ERK1/2 MAPK (rabbit anti-mouse/human, #9102) from Cell Signaling, Inc. (Danvers, MA). Inhibitors used include: oseltamivir phosphate (Santa Cruz Biotechnology, Dallas, TX); ERK MAPK inhibitor U0126, JNK MAPK inhibitor SP600125, and PI3K inhibitor wortmannin (Calbiochem, San Diego, CA); p38 MAPK inhibitor SB203580 (AdipoGen, San Diego, CA). Ultra-pure Lipopolysaccharide (LPS) (Invivogen, San Diego, CA) was used for MC stimulation.

Mice and lupus serum collection

All animal studies and methods of euthanasia were approved by the local Institutional Animal Care and Use Committee (IACUC) and complied with ARRIVE guidelines. Breeding pairs of MRL/lpr mice were purchased (Stock #000485, Jackson Laboratory, Bar Harbor, ME) and bred in-house. Breeding pairs for C57BL/6 Neu1^{+/-} mice³⁶ were generously provided by Dr. Alessandra d'Azzo from St. Jude's Children's Research Hospital and bred in-house. Mice were maintained on a 12 h light-dark cycle in groups of 3-5 per cage in a pathogen free environment with access to food and water ad libitum. Blood was collected from 3-4 MRL/lpr or C57BL/6 female or male mice at 16-18 weeks of age and pooled by sex. Serum was collected after centrifugation of coagulated blood and is referred to as lupus serum (LS) and B6 serum (B6) in the text and figures. Each pooled serum collection was tested for endotoxin using Pierce Chromogenic Endotoxin Quantification Kit (Thermo Scientific, Rockford, IL) and only serum collections with undetectable levels of endotoxin were used for experiments. Both female and male serum stimulated significant cytokine production.

Generation and culturing of primary mesangial cell lines

Two independent mouse primary mesangial cell lines (MCs), established using glomeruli pooled from 3-4 six-week-old female MRL/lpr mice was described and characterized previously ¹², were used for experiments.

Kidney glomeruli were isolated from 6-week-old female C57BL/6 Neu1^{+/+} and C57BL/6 Neu1^{+/-} mice for generation and characterization of two independent primary cell lines (B6 MCs) as described previously ¹².

Since Neu1^{-/-} mice develop a kidney phenotype ³⁶ we isolated MCs from Neu1^{+/-} mice to avoid potential confounding factors in using Neu1^{-/-} MCs. Neu1 genotype of each B6 MC line was confirmed via PCR as described previously ³⁶. Cultures were routinely tested for mycoplasma (Applied StemCell, Inc, Milpitas, CA) and only mycoplasma negative cultures were used in experiments. Cells were grown in a 5% CO₂ atmosphere at 37°C in MEM/D-valine medium with 20% fetal bovine serum, 1% penicillin/streptomycin and ITS supplement (5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml sodium selenite) (GenDEPOT, Houston, TX; Sigma-Aldrich, St. Louis, MO) and used at passages 6-9. Cells were serum starved overnight prior to stimulation with lupus serum, B6 serum, or LPS at the concentrations indicated and for the time indicated in the figures or figure legends. Results from each cell line showed similar trends with data presented from one line. Extent of cytokine release varied from experiment to experiment in response to lupus serum depending on the pooled serum collection used. All statistically significant changes reported using lupus serum were calculated after combining replicate experiments and adjusting for serum “batch” differences as described in the statistical analyses section below and presented graphs are from a representative experiment.

Cells were pre-treated with 500 µM oseltamivir phosphate (OP), an inhibitor of NEU activity, for 16 hrs, 40 µg/ml antibodies for 2 hrs, or 10 µM MAPK/PI3K inhibitors for 2 hrs, or relevant vehicle/IgG for the same time prior to stimulation. Our previously published data demonstrated a dose-dependent inhibition of IL-6 release by MRL/lpr MCs using 125-500 µM OP ¹². A dose of 125 µM inhibited IL-6 by >50% in response to stimulation with lupus serum and 500 µM provided >90% inhibition and was similar to what was observed in other cell types ^{32, 37}. Thus, we chose to use a concentration that maximally inhibited IL-6. Importantly, this concentration of OP was not toxic to the cells as assessed by trypan blue exclusion and cell counting, and measuring total protein content, which varied less than 1% across treatments in all experiments. This was confirmed using a cell viability MTT assay following manufacturer’s instructions (BioAssay Systems, Hayward, CA) (supplemental Fig. S1).

Neuraminidase activity assay

Primary MRL/lpr MCs (20,000 cells per well) were grown in a CoStar 3603 black 96-well clear-bottom plate, serum-starved, pre-treated with 500 μ M OP, and stimulated with 10% lupus serum or 5ng/ml LPS for 3 hrs. Primary B6 Neu1^{+/+} and B6 Neu1^{+/-} MCs (20,000 cells per well) were grown in a CoStar 3603 black 96-well clear-bottom plate, and serum starved prior to stimulation with 20% lupus serum for 3 hrs. After incubation, media was collected, and cells were washed with 37°C warmed sterile filtered Tris-buffered saline (TBS) pH 7.4. Cells were incubated with 15 μ M substrate 2' (4-methylumbelliferyl)- α -D-N-acetylneuraminic acid (4MU-NANA) at 37°C in TBS (Sigma Aldrich; St. Louis, MO). Fluorescence intensity (excitation: 365 nm, emission: 460 nm) in the absence of substrate (baseline) and after a 15' incubation with substrate was measured using a SpectraMax i3c fluorometer with SoftMax Pro 6 software (Molecular Devices, San Jose, CA). NEU activity is presented as relative fluorescence units (RFU) per 2×10^4 cells. Total protein content was consistent between untreated and treated cells. Significant differences were determined by one-way ANOVA with Tukey's multiple comparisons test. Adjusted p-values are presented.

FlexMap 3D and ELISA analyses

To determine cytokines and chemokines in addition to IL-6 potentially mediated by NEU activity, primary MRL/lpr MCs were incubated with increasing concentrations of lupus serum (5, 10 and 20%), 20% C57BL/6 serum, or 10% lupus serum following pre-treatment with increasing concentrations of OP (125, 250, and 500 μ M) (as described above). Cells were pre-treated with OP 16 hrs prior to addition of lupus serum for 6 hrs. MC supernatants were analyzed by multiplex bead array analysis using a FlexMap3D platform (Luminex, Austin, TX) and Cytokine Mouse Magnetic 20-Plex Panel kit (Invitrogen/ThermoFisher Scientific, Waltham, MA). The FlexMap3D results were confirmed by individual commercially available enzyme-linked immunoassay (ELISA) detection kits to measure IL-6 (BioLegend, San Diego, CA), GM-CSF (R&D systems, Minneapolis, MN), and MIP1 α (R&D systems, Minneapolis, MN) in primary MC supernatants according to the manufacturer's instructions. Protein concentration of cells was measured using a Micro BCA Protein Assay Kit (Thermo Scientific, Rockford, IL) and used to normalize cytokine levels with final concentrations adjusted to an equivalent of 25 μ g of protein for comparison across experiments.

Real-time PCR

Total RNA was extracted from primary MCs using the RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, and cDNA was reversely transcribed with 0.5–1 µg RNA using the iScript cDNA synthesis kit as described in the kit (Biorad, Hercules, CA). The gene expression of *Il-6*, *Gm-csf*, *Mip1α* and housekeeping gene *β-actin* was evaluated by quantitative RT-PCR using the LightCycler 480 SYBR Green 1 Master kit and LightCycler 480 II (Roche, Indianapolis, IN) and oligos: *mIl-6* Forward 5'-TAGTCCTTCCTACCCCAATTTC-3' and Reverse 5'-TTGGTCCTTAGCCACTCCTTC-3'; *mGm-csf* Forward 5'-GGCCTTGGAAGCATGTAGAGG-3' and Reverse 5'-GGAGAACTCGTTAGAGACGACTT-3'; *mMip1α* Forward 5'-ACTGCCTGCTGCTTCTCCTACA-3' and Reverse 5'-AGGAAAATGACACCTGGCTGG-3'; *mNeu1* Forward 5'-ACGATGTAGACACAGGGATAGTG-3' and Reverse 5'-GTCGTCCTTACTCCAAACCAAC-3'; *mβ-actin* 5'-AGATTACTGCTCTGGCTCCTAG-3' and Reverse 5'-CCTGCTTGCTGATCCACATC-3'. The PCR mixture was heated initially at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 10 s and combined annealing/extension at 60 °C for 30 s. All reactions were performed in triplicate and normalized to *β-actin*. Gene expression data analysis was performed by using the comparative threshold cycle (CT) method as performed previously ¹².

Immunoblotting

Mesangial cell extracts were prepared by incubating cells with RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% NaDeoxycholate, 0.1% SDS, 25 mM DTT, 10 mM EDTA, 1 mM PMSF). After centrifugation at 4°C for 10 minutes at 14,000 rpm, the supernatant was collected and protein concentration was determined using a micro BCA assay (Pierce/Thermo Scientific, Rockford, IL). Samples (20–40 µg per well) were electrophoresed in a 4-20% Criterion TGX gel (Biorad, Hercules, CA), and transferred to a polyvinylidene difluoride membrane. Total and phosphorylated MAPK were immunoblotted with antibodies to total and phosphorylated forms of p38 and ERK MAPK and detected with an anti-rabbit biotin antibody (Thermo Scientific, Rockford, IL) followed by an AlexaFluor-647 streptavidin-conjugated antibody (Life Technologies, Grand Island, NY). Blots were scanned using Odyssey Infrared Imaging system and software (LI-COR, Lincoln, NE) and band density was quantified using NIH ImageJ. The results are presented as the ratios of phosphorylated to total p38 or ERK MAPK. An additional band was observed for total ERK in the stimulated cells, which appears to be a modification of the lowest molecular weight band resulting in a slower migrating band. Three independent experiments were performed with independent pooled collections of lupus serum. Increased phosphorylation of ERK and p38 was consistently observed in response to all lupus serum

collections with the extent of phosphorylation varying depending on the serum used. Blots presented are representative of all three experiments.

N-glycan analyses

Sialylated glycoprotein N-glycans were measured in intact cells that were grown on glass slides and cultured with lupus serum in the absence (6 replicate wells) or presence of OP (3 replicate wells). Cells were incubated with OP for 16 hrs and media replaced with 10% lupus serum for 3 hrs. Following stimulation, cells were prepared for N-glycan profiling as described previously³⁸. Briefly, the array was washed in cold PBS and fixed in neutral buffered formalin followed by delipidation with three replicates of Carnoy's Solution (10% glacial acetic acid, 30% chloroform, 60% 200 proof ethanol) for three minutes. Deglycosylation was performed by spraying each array with PNGase F Prime (N-zyme Scientifics) using an M3 TM-Sprayer (HTX Technologies) with 10 passes at 25 μ L/min, 1200 mm/s, 45°C, 3 mm spacing between passes with 10 psi nitrogen gas and incubated for 2 hours at 37°C and $\geq$ 80% relative humidity. MALDI matrix α -cyano-4-hydroxycinnamic acid (CHCA, Sigma; 50% acetonitrile/0.1% trifluoroacetic acid) was sprayed onto the cells with an M3 TM-Sprayer (HTX Technologies) using 10 passes at 70 μ L/min, 1300 mm/s, 79°C, 2.5 mm spacing between passes and 10 psi nitrogen gas. Two passes of ammonium phosphate monobasic (5 mM) were applied using same parameters as for matrix. N-glycans were profiled using a Fourier Transform Ion Cyclotron Resonance Mass Spectrometer (FT-ICR; 7 Tesla solariXTM, Bruker Scientific, LLC) equipped with a MALDI source. Transients of 512 kiloword were acquired in broadband positive ion mode over m/z 500-5,000 with a calculated on-tissue mass resolution at full width half maximum of 81,000 at m/z 1400. Each pixel consisted of 1200 laser shots rastered over a 500 μ m diameter area. A lockmass on primary N-glycan peak m/z 1663.5814 (composition Hex5HexNAc4 + 1Na) was used to maintain mass accuracy during acquisition. N-glycoform assignment to regulated N-glycans was made by high mass accuracy match ($\pm$ 2 ppm) to the singly charged M+Na peak using GlycoWorkbench³⁹. Data presented are for quantified peak intensities of sialylated N-glycans after subtracting background levels obtained from wells cultured with media only (no cells).

Statistical analyses

Association between treatment or duration of exposure to lupus serum or LPS in the absence or presence of OP with outcomes (e.g. protein, mRNA levels, GSLs) were evaluated using a linear mixed model approach.

Models with one treatment variable included a fixed effect for treatment and models that included treatment and time included fixed effects for treatment, time, and the treatment X time interactions. All experiments were performed at least twice with similar results as indicated in the figure legends. Data were combined for the statistical analyses. Since stimulatory activity of the lupus serum varied from batch to batch, the graphs presented in Figs. 1-4 and Fig. 7A are from a single representative experiment but include p-values calculated across experiments. For example, IL-6 secretion (as in Fig. 1C) increased by ~1,200-5,100 pg/ml in response to a 3 hr incubation with 10% lupus serum compared to Control (~20-100 pg/ml) depending on serum batch. Thus, all models included a random batch effect to account for correlation between experimental results from the same batch. Model assumptions were checked graphically and transformations were considered as needed. Pairwise differences between groups were evaluated using linear contrasts and a Bonferroni correction was applied to adjust for multiple comparisons. All analyses were conducted in SAS v. 9.4 (SAS Institute, Cary, NC). Significant differences in nineteen sialylated N-glycans between lupus serum-stimulated cells pre-treated with vehicle (water) or OP were evaluated using a two-sample t-test. Both adjusted and unadjusted p-values were considered with adjusted p-values provided on the graph. Four additional N-glycans had background subtracted values less than zero and were excluded from the analysis. Global p-values are provided for analyzing lupus serum or LPS dose response of cytokine production (Figs. 1A-B, 5A-C). Error bars represent standard deviation for the representative experiments presented in figures. Adjusted p-values are presented on all graphs.

RESULTS

Lupus serum stimulates cytokine secretion and mRNA expression in lupus prone MCs

We previously demonstrated a dose-dependent significant release of IL-6 in response to lupus serum¹². To better define the effects of lupus serum in stimulating lupus prone MCs, Multiplex FlexMap3D bead array analysis was used to measure a panel of twenty cytokines. MRL/lpr MCs were cultured for 6 hrs in the presence of 5, 10 or 20% lupus serum or 20% serum taken from age-matched healthy non-autoimmune prone C57BL/6 (B6) mice, or 10% lupus serum after pre-treating with 125, 250, or 500 μ M oseltamivir phosphate (OP), an inhibitor of NEU activity. Secretion of seven cytokines (IL-6, IL-12, IL-17, MIP1 α , GM-CSF, IFN γ , and IP-10) showed a dose-dependent increase of at least four-fold after addition of lupus serum and a dose-dependent decrease in the presence of OP. Individual ELISAs confirmed significant dose-dependent secretion of GM-CSF (Fig. 1A, p=0.013) and MIP1 α (Fig. 1B, p<0.001) in response to lupus serum (in addition to IL-6

¹²). We then analyzed the temporal release of these cytokines in response to 10% lupus serum stimulation. Media was collected from lupus MCs stimulated with lupus serum for 0.5, 1, 3, and 6 hrs. Significantly increased secretion of all three cytokines was observed by 3 hrs following addition of lupus serum (Fig. 1C-E). Message levels of *Il-6* (Fig. 1F) and *Gm-csf* (Fig. 1G) were also significantly increased following lupus serum stimulation with significant increases in *Gm-csf* mRNA levels being observed within 1 hr of lupus serum stimulation (Fig. 1G). *Mip1α* mRNA levels were undetectable in unstimulated cells and remained at the threshold of detection (>32 cycles) after stimulation making quantification unreliable. Thus, we were unable to conclude with any degree of certainty if *Mip1α* expression was increased following lupus serum stimulation. *Neu1* message levels in response to lupus serum significantly increased at 1 hr, but had significantly decreased at 3 hrs (Fig. 1H). *Neu1* message remained slightly, but not significantly, lower at 6 hrs. *Neu3* mRNA was undetectable at all time points. These results demonstrate that expression of *Il-6*, *Gm-csf*, and *Neu1* are elevated prior to or around the same time as cytokine release in response to lupus serum.

NEU activity mediates cytokine secretion from lupus serum stimulated primary lupus prone MCs

Our previous results demonstrated IL-6 secretion by MRL/lpr MCs in response to both HA-IgG and lupus serum was dose-dependently inhibited by OP ¹². These results strongly suggested IL-6 release by MCs is mediated by NEU activity. To determine if secretion of GM-CSF and MIP1α are also mediated by NEU activity in response to lupus serum, MCs were pre-treated with 500 μM OP, which prevented >90% of IL-6 release¹², or vehicle (water) and stimulated with lupus serum for 6 hrs. This experiment confirmed all three cytokines were significantly decreased when MCs were pre-treated with OP prior to lupus serum stimulation (Fig. 2A-C). OP treatment of MRL/lpr MCs blocked the increase in *Il-6* mRNA levels, actually further increased *Gm-csf* mRNA levels in response to lupus serum, and had no effect on *Neu1* mRNA levels (Fig. 2D). A live-cell activity assay demonstrated that NEU activity significantly increased in response to lupus serum (Fig. 2E) despite a significant decrease in *Neu1* expression (Fig. 1H) 3 hr after lupus serum stimulation, suggesting a potential negative feedback loop. We also confirmed that the lupus serum-stimulated NEU activity was significantly inhibited by OP. Together these results suggest NEU activity mediates secretion of at least three cytokines by lupus prone MCs and specifically mediates IL-6 secretion by promoting its expression at the message level in response to lupus serum.

To confirm the effects of OP on cytokine release are due to blocking NEU activity specifically and not due to an off-target effect, we cultured primary MCs from C57BL/6 mice that are wild-type for *Neu1* (B6 *Neu1*^{+/+})

or heterozygote for Neu1 (B6 Neu1^{+/-}) and stimulated with serum from MRL/lpr mice (lupus serum) or age-matched C57BL/6 mice. The Neu1^{+/-} B6 MCs had significantly reduced NEU activity compared to the B6 Neu1^{+/+} MCs (Fig. 3A) and significantly reduced IL-6 secretion (Fig. 3B) at baseline and following stimulation with lupus serum. Serum from age-matched C57BL/6 mice (B6 serum) did not stimulate IL-6 secretion (Fig. 3B) as previously demonstrated with MRL/lpr MCs¹². These results are in agreement with our previous results that showed reducing *Neu1* expression with siRNA in heat aggregated IgG-stimulated MRL/lpr MCs significantly decreased IL-6 secretion¹². Together, these results support those observed in Fig. 2 with OP, indicating NEU activity (and specifically NEU1) plays a role in mediating cytokine release from MCs.

Lupus serum stimulates IL-6 secretion from MRL/lpr MCs through TLR4/MD2

The secretion of pro-inflammatory cytokines including IL-6, GM-CSF, and MIP1 α can be directly stimulated through signaling pathways in MCs following ligand binding to cell surface proteins such as Fc γ receptors (Fc γ Rs), toll like receptors (TLRs), annexin II (AnxA2), and TNF receptors (TNFRs)⁴⁰⁻⁴⁴. Therefore, the role of these proteins in lupus serum-stimulated primary MCs was investigated. MCs were incubated with neutralizing antibodies for cell-surface TLRs and Fc γ Rs to block their interaction with putative ligands prior to addition of lupus serum. TLR4/MD2 neutralizing antibody significantly inhibited lupus serum-stimulated IL-6 secretion (Fig. 4A, $p < 0.001$). Secretion of GM-CSF was slightly, but not significantly, reduced (Fig. 4B), and MIP1 α was not significantly impacted (Fig. 4C) by anti-TLR4/MD2 antibodies. Antibodies to Fc γ Rs (Fig. 4), and TLR4 alone, AnxA2, and TNFRs, (data not shown) failed to reduce IL-6, GM-CSF, or MIP1 α secretion. The lack of effect by multiple other antibodies suggest strongly a specific effect of anti-TLR4/MD2 antibodies and that lupus serum stimulates IL-6 secretion from lupus prone MCs in part through TLR4 and MD2.

NEU activity mediates IL-6 and GM-CSF secretion through TLR4 activation in MRL/lpr MCs

To confirm NEU activity mediates cytokine release specifically through a TLR4 pathway in MRL/lpr MCs, we isolated this pathway by stimulating the cells with the TLR4 ligand LPS. As expected, LPS stimulated significant secretion of IL-6 (Fig. 5A, $p = 0.002$), GM-CSF (Fig. 5B, $p = 0.013$), and MIP1 α (Fig. 5C, $p = 0.002$). Increasing the concentration of LPS did not further increase the levels observed at 5 ng/ml indicating release was maximal at this concentration. Thus, 5 ng/ml LPS was used to stimulate cells for all subsequent

experiments. Secretion and mRNA expression of these cytokines by LPS followed a similar temporal trend as that observed following lupus serum stimulation. Significant increases in all three cytokines were observed in the media within 3 hrs of LPS addition (Fig. 5D-F). Message levels of *Il-6* (Fig. 5G), *Gm-csf* (Fig. 5H), and *Neu1* (Fig. 5I) increased within 1 hr of stimulation. Although *Neu1* message levels did not remain elevated, levels were not significantly decreased at 3 hr as observed with lupus serum in Fig. 1H. As with lupus serum stimulation, *Mip1α* mRNA levels were essentially undetectable after LPS stimulation. Importantly, inhibiting NEU activity with the inhibitor OP, significantly reduced LPS-stimulated secretion of IL-6, GM-CSF, and MIP1α (Fig. 6A-C), and mRNA expression of *Il-6* and *Gm-csf* (Fig. 6D). Whereas *Gm-csf* mRNA levels were increased by OP treatment in lupus serum-stimulated MCs, they were decreased by OP in LPS-stimulated MCs. A live-cell NEU activity assay demonstrated significant NEU activity in response to LPS stimulation, which was significantly blocked by OP (Fig. 6E). Addition of TLR4/MD2 neutralizing antibodies significantly blocked LPS-stimulated IL-6 and GM-CSF secretion, but had no effect on MIP1α secretion (Fig. 6F). Together, these novel observations suggest strongly that NEU-mediated secretion of IL-6 and GM-CSF from lupus prone MCs occurs in part through TLR4 signaling in lupus prone MCs, and that NEU may directly mediate TLR4 signaling of IL-6 secretion.

NEU activity mediates TLR4-MAPK signaling in secretion of IL-6 and GM-CSF from MRL/lpr MCs

Since the TLR4 receptor can signal through MAPK and PI3K pathways, we wanted to determine if MAPK (ERK, JNK, p38) or PI3K pathways play a role in NEU activity-mediated release of IL-6 in MCs. Inhibitors for each pathway were incubated with the cells prior to stimulation and included ERK, p38, and JNK inhibitors U0126, SB203580, and SP600125, respectively, and PI3K inhibitor wortmannin. Only ERK and p38 inhibitors significantly reduced IL-6 secretion from both lupus serum-stimulated (Fig. 7A, $p < 0.001$ for both inhibitors) and LPS-stimulated (Fig. 7B, $p < 0.001$ for both inhibitors) MCs. Inhibiting ERK also significantly reduced GM-CSF secretion from lupus serum-stimulated (Fig. 7A, $p < 0.001$) and LPS-stimulated (Fig. 7B, $p < 0.001$) MCs. None of the inhibitors reduced MIP1α secretion from MCs when stimulated with either lupus serum or LPS. Interestingly, JNK and PI3K inhibition significantly increased GM-CSF ($p < 0.001$ for both), and PI3K inhibition significantly increased MIP1α secretion ($p < 0.001$) in LPS-stimulated cells (Fig. 7B).

To confirm a role for ERK or p38 MAPK signaling in NEU activity-mediated TLR4 signaling, MRL/lpr primary MCs were incubated without or with OP and stimulated with lupus serum or LPS. Activation of both ERK and p38 was increased as indicated by the increase of their phosphorylated forms in response to lupus

serum and reduced by the NEU activity inhibitor OP (Fig. 7C). Similarly, phosphorylation of ERK and p38 was observed following LPS stimulation, and reduced by inhibiting NEU activity with OP (Fig. 7D). Although the extent of phosphorylation of ERK and p38 in response to lupus serum varied depending on the lupus serum collection that was used, phosphorylation was consistently observed across experiments for both ERK and p38, as was reduction by OP in both lupus serum-stimulated and LPS-stimulated MCs. Across experiments, semi-quantitative measurement of band intensities showed a 2.4- to 7-fold or 2- to 3.3-fold increase in the ratio of phosphorylated p38 to total p38 (P-p38/T-p38) in response to lupus serum or LPS, respectively, compared to vehicle. Similarly, a 2- to 4.3-fold or 2- to 2.3-fold increase in P-ERK/T-ERK was observed in response to lupus serum or LPS, respectively. In all experiments, pre-treatment with OP prior to stimulation reduced P-p38/T-p38 or P-ERK/T-ERK by 40-60% compared to stimulated cells. Together, these results suggest NEU activity mediates IL-6 and GM-CSF cytokine release through a TLR4-ERK/p38 MAPK pathway in lupus prone MCs.

Inhibiting NEU activity significantly increases sialic acid-containing glycoproteins in lupus serum-stimulated MCs.

Sialic acid-containing glycolipids and glycoproteins serve as substrates of NEUs and removal of sialic acids (desialylation) plays an important role in TLR4 activation and signaling in other cell types⁴⁵⁻⁴⁷. We recently showed that in MRL/lpr MCs the overall level of sialic acid-containing glycosphingolipid GM3s are significantly increased in the presence of OP⁴⁸, suggesting OP indeed blocks NEU activity against glycolipids. Here, we profiled N-linked glycoproteins (N-glycans) to determine if blocking NEU activity with OP increases sialic acid-containing N-linked glycoproteins (N-glycans), such as TLR4 and MD2, in lupus-serum stimulated MCs. The intensities of 19 sialylated N-glycans clearly detectable after background subtraction were measured in intact MRL/lpr MCs that were pre-treated with OP or vehicle and then stimulated with 10% lupus serum for 3 hr. In the cells pre-treated with OP the intensity of ~14 of the 19 detected sialylated N-glycans increased in intensity, with five N-glycans being significantly increased, when NEU activity was reduced (Fig. 8).

Structures of two glycans (m/z 2122 and m/z 2853) that exhibited significantly increased intensity are shown on the graph. These results demonstrate OP blocked the activity of NEU in removing sialic acids from a subset of N-glycans. Together our results suggest NEU activity may mediate cytokine release in part by desialylating glycoproteins, such as TLR4 which we demonstrated is involved in IL-6 release, in lupus serum-stimulated MRL/lpr MCs.

DISCUSSION

One of the earliest events in the pathogenesis of lupus nephritis is the deposition of immune complexes to promote cytokine production by mesangial cells (MCs) and podocytes. IL-6 and other cytokines released by resident renal cells promote the infiltration of immune cells, further stimulation of cytokine production by and proliferation of MCs, and subsequent tissue damage. Blocking or reducing IL-6 in lupus prone mice attenuated disease^{7, 10, 11}; however, anti-IL-6 treatment of lupus patients with class III or IV nephritis in a small clinical trial was not efficacious⁴⁹. Delineating the precise mechanisms by which IL-6 levels are increased in LN is needed. A recent study demonstrated IL-6 is specifically produced by glomerular MCs in nephritic mice of the NZM2328 lupus strain⁵⁰, and we previously demonstrated MCs from MRL/lpr lupus prone mice release IL-6 and MCP-1 following stimulation with an immune complex mimic (heat-aggregated IgG) or lupus serum¹². Previously published data and our results here suggest strongly that NEU activity, and specifically NEU1, play a role in promoting cytokine release from MCs^{12, 48}. This includes increased NEU activity and cytokine secretion in response to a variety of stimuli (HA-IgG, lupus serum, LPS) that is significantly reduced by treating cells with the NEU inhibitor OP. In our live cell assay, OP was observed to inhibit NEU activity by <20%, which may specifically reflect NEU1 activity since OP was reported to have specificity for NEU1^{32, 37, 51}. Clearly, reducing NEU activity by <20% in MRL/lpr MCs and by ~35% in B6 Neu1^{+/-} MCs had significant effects on cytokine release emphasizing the importance of altering NEU activity levels in the function of MCs. Additional evidence that NEU1 is the NEU primarily responsible for the observed effects include inhibition of IL-6 release by Neu1 siRNA in MRL/lpr MCs¹², the increase in *Neu1* mRNA levels within 1 hr of lupus serum or LPS stimulation (Figs. 1H and 5I), and the significantly reduced IL-6 release by B6 MCs heterozygote for Neu1 in response to lupus serum (Fig. 3B). Here we also provide data supporting that NEU activity promotes IL-6 secretion through a TLR4-MAPK p38 or ERK signaling pathway in response to a circulating lupus factor(s). This is likely a direct mediation by NEU activity since both IL-6 protein and *Il-6* mRNA levels are significantly reduced when NEU activity is blocked.

The pro-inflammatory activity of lupus serum is largely thought to require the presence of circulating immune complexes and their deposition in the kidney likely plays a major role in stimulation of renal resident cells. Autoantibodies and immune complexes with the ability to activate FcγRs and TLRs promote increased production of inflammatory mediators. Our previous studies showed colocalization of NEU1 and NEU3 with

IgG in cultured primary MCs stimulated with exogenously added heat-aggregated IgG and in the glomeruli of kidney sections from nephritic MRL/lpr mice ¹². Results from our current study indicate FcγRs, which bind IgG, are not required for NEU activity-mediated release of IL-6 in response to lupus serum. These results agree with previous studies demonstrating that MRL/lpr mice lacking the Fcγ chain still develop renal disease ⁵², and FcγRs were dispensable for activation of MRL/lpr ⁴⁰ and human ⁵³ MCs by pathogenic anti-DNA antibodies. Instead, we demonstrated lupus serum-stimulated IL-6 secretion from MRL/lpr MCs was significantly decreased when TLR4 was blocked and that LPS-stimulated TLR4 signaling of IL-6 secretion was significantly decreased when NEU activity was blocked. Although TLR4 is not readily detected in MCs from healthy non-autoimmune mice, it is present in MCs from MRL/lpr lupus mice ⁴¹, and TLR4 deficiency in another lupus prone mouse strain decreased renal disease ⁵⁴. Together these results support a role for TLR4 in NEU activity-mediated IL-6 expression and secretion in response to lupus serum.

NEU activity also mediated GM-CSF and MIP1α secretion from lupus prone MCs. Blocking TLR4 in lupus serum-stimulated cells resulted in a slight reduction in GM-CSF secretion and blocking TLR4 in LPS-stimulated cells resulted in a significant reduction of GM-CSF. Furthermore, blocking NEU activity significantly reduced *Gm-csf* message levels in LPS-stimulated, but not lupus serum-stimulated cells. These data suggest TLR4 plays a role, but other receptors may also be involved in NEU activity-mediated GM-CSF secretion in response to lupus serum. MIP1α was not decreased when TLR4 signaling was blocked, indicating NEU activity may mediate MIP1α secretion indirectly or through a mechanism other than TLR4 signaling.

NEU activity can mediate cell signaling by removing sialic acids (desialylating) from glycolipids or glycoproteins. MRL/lpr MCs treated with OP results in a highly significant accumulation of sialylated ganglioside GM3 ⁴⁸ and an increase in a subset of sialylated N-glycans (Fig. 8). These results indicate OP blocks the activity of NEU in removing sialic acids from a subset of glycolipids and glycoproteins. OP did not prevent desialylation of N-glycans levels globally. This is not surprising given that OP was shown to preferentially inhibit NEU1 activity in other cell types ^{32, 37, 51}. Mammalian NEUs exhibit preferences for specific glycolipids and/or glycoproteins with NEU1 shown to be more active toward glycoproteins ⁵⁵. Based on these collective results, we postulate that NEU1 desialylates glycoproteins, likely TLR4 or MD2, to promote TLR4-MAPK-IL-6 signaling.

Sialic acids (SAs) mask receptors from ligands to prevent signaling⁵⁶. TLR4 and MD2, both of which are required for optimal TLR4 signaling, contain multiple N-glycans required for function that can be sialylated. TLR4 diffuses into lipid rafts upon LPS stimulation to propagate signaling of gene expression⁵⁷. In other cell types, removal of SAs by the action of NEUs enhanced binding and subsequent signaling through TLR4^{31, 47, 58, 59}. This TLR4 activation was shown to trigger NEU1 translocation to the cell surface to desialylate TLR4 and MD2 and enhance TLR4 dimerization and signaling^{46, 47, 60}. *In vivo*, increasing sialylation by treating mice with exogenous SAs reduced TLR4 activation and neutralized LPS toxicity-induced renal injury⁶¹. We observed that antibodies to both TLR4 and MD2, but not to TLR4 alone, block MC response to lupus serum. Unfortunately, the N-glycan structures of TLR4 are unknown. Thus, we were unable to determine if any of the N-glycans identified in Fig. 8 belong to TLR4. Alternatively, or additionally, the function of NEU activity in mediating IL-6 production by MCs may be through the desialylation of glycolipids involved in signaling pathways. Sialic acid-containing gangliosides present in cell membranes, such as GM3, impact cell functions by organizing lipid rafts to modulate receptor-mediated signal transduction⁶². Increasing GM3, the simplest sialic acid-containing ganglioside from which all other gangliosides are generated, prevents MC activation and proliferation⁶³. Since proliferation is inhibited when GM3 is increased in MCs⁶³, the observed significant decrease in IL-6 secretion when NEU activity was blocked with OP may be a result of increased GM3 levels⁴⁸. Extensive additional studies are required to determine the expression and activity of individual sialyltransferases and NEUs in stimulated MCs and further define the mechanisms contributing to the production of IL-6 and other proinflammatory cytokines.

One limitation in our study is the specificity of OP, which was developed as an inhibitor of viral NEU activity. Unfortunately, specific inhibitors of mammalian NEUs are not currently commercially available. There are conflicting reports in the literature as to whether OP is effective in blocking the activity of mammalian NEUs. Studies that reported oseltamivir failed to block mammalian NEU activity largely used the carboxylate form (OC), not the phosphate form (OP), on homogenates from cells overexpressing mammalian NEUs or on purified NEU preparations^{64, 65}, which may account for some of the conflicting observations. Studies that demonstrated oseltamivir blocks mammalian NEU activity used live cells treated with 200-500 μ M OP concentrations and analyzed endogenous NEU activity^{32-34, 37, 66, 67}. These conditions are similar to what we used in this study and in our previous study¹², which was not toxic to the cells. We also showed reducing NEU activity by reducing *Neu1* expression in C57BL/6 MCs (*Neu1*^{+/-}) resulted in a significant reduction in IL-6 secretion, further supporting NEU activity, and NEU1 specifically, mediates IL-6 secretion. However, the

possibility that some observed effects of OP are due to off-target effects cannot be completely ruled out. Although OP only reduced NEU activity in our live cell assay by ~20%, it was sufficient to have a significant effect on cytokine release. In addition, activation of signaling pathways and the role of NEU activity is likely to change in response to the factors present in lupus serum. Circulating pathogenic disease factors may fluctuate depending on disease stage/state of the mice from which the lupus serum was obtained, which can vary from mouse to mouse. The differences in presence/absence or concentration of these factors likely influence the pathways activated and the role of NEU activity. Indeed, differences were observed in the extent of cytokine release in response to lupus serum across serum collections from 16-18-week-old mice MRL/lpr mice. While using LPS allowed us to isolate TLR4 signaling to demonstrate a role for NEU activity in this pathway, future studies will be required to identify the specific circulating factors involved temporally.

In summary, we demonstrated stimulation of lupus prone primary MCs with lupus serum or LPS results in the secretion of IL-6, GM-CSF, and MIP1 α and is mediated in part by NEU activity, most likely NEU1 activity. We further showed NEU activity-mediated IL-6 production likely occurs through p38/ERK MAPK upregulation of *Il-6* expression following TLR4 activation, and may involve desialylation of glycans in response to circulating lupus factor(s). These novel observations will provide a better understanding of proinflammatory mechanisms and lays the foundation to identify additional putative therapeutic targets in lupus nephritis.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

DATA AVAILABILITY

Data or materials generated by this study are available upon request.

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FIGURES

Fig. 1. Lupus Serum stimulates expression and secretion of IL-6 and GM-CSF, and secretion of MIP1 α from lupus prone primary MCs. GM-CSF (A), and MIP1 α (B) were measured by individual ELISAs in the media of primary MRL/lpr MCs cultured for 6 hrs in the absence (Control) or presence of increasing concentrations of lupus serum. IL-6 (C), GM-CSF (D), and MIP1 α (E) were measured by individual ELISAs in the media of primary MRL/lpr MCs cultured for the indicated time in the absence (Control) or presence of 10% lupus serum. *Il-6* (F), *Gm-csf* (G), and *Neu1* (H) mRNA expression were measured by real-time RTPCR in primary MRL/lpr MCs cultured for 0.5-6 hrs in the absence (Control) or presence of 10% lupus serum. Experiments were performed three times in A and B and two times in C-H with independent serum collections. Global p-values in A-B and individual p-values between Control and Lupus Serum at each time point for C-H were calculated across replicate experiments as described in the statistical analyses section and provided on graphs of data from representative experiments.

Fig. 2. Inhibiting NEU activity significantly reduces lupus serum-stimulated secretion of IL-6, GM-CSF, and MIP1 α , and expression of *Il-6*. Primary MRL/lpr MCs were cultured in the absence or presence of 10% lupus serum (LS) and 500 μ M NEU inhibitor oseltamivir phosphate (OP). IL-6 (A), GM-CSF (B), and MIP1 α (C) were measured by individual ELISAs in the media 6 hrs after addition of LS. D) *Il-6*, *Gm-csf*, and *Neu1* mRNA expression were measured by real-time RTPCR 6 hrs after addition of LS. Control, treated with vehicle (water) only (no OP or LS). E) NEU activity by addition of substrate to live cells was measured after stimulation with LS for 3 hrs. Experiments were performed three times in A, B, D, and E and twice in C with independent serum collections. P-values were calculated across replicate experiments as described in the statistical analyses section and provided on graphs of data from representative experiments.

Fig. 3. Genetically reducing *Neu1* significantly reduces basal and lupus serum-stimulated NEU activity and lupus serum-stimulated secretion of IL-6. A) Primary C57BL/6 *Neu1*^{+/+} and *Neu1*^{+/-} MCs were cultured in the absence (Control) or presence of 20% lupus serum (LS) for 3 hrs. NEU activity was measured after addition of NEU substrate to live cells. B) Primary C57BL/6 *Neu1*^{+/+} *Neu1*^{+/-} MCs were cultured in the absence (Control) or presence of 20% or 40% lupus serum (LS) or 40% C57BL/6 (B6) serum. IL-6 was measured by ELISA in the media 24 hrs after addition of serum. Experiments were performed four times in A and twice in B with at least two independent serum collections. P-values were calculated across replicate

experiments as described in the statistical analyses section and provided on graphs of data from representative experiments.

Fig. 4. Blocking TLR4 significantly decreased lupus serum-stimulated IL-6 secretion. Primary MRL/lpr MCs were cultured with 10% lupus serum for 6 hrs following pre-treatment without or with the indicated antibodies (Ab). IL-6 (A), GM-CSF (B), and MIP1 α (C) were measured in the media by ELISA. Secretion in presence of LS and No Ab was set to 1 for each cytokine and relative fold change in presence of Ab presented. Experiments were performed three times in A-B and twice in C with at least two different serum collections and fold change averaged across experiments. Absolute levels of all three cytokines across replicate experiments were similar to levels observed in Figs. 1-2 in response to lupus serum. Individual p-values are provided on the graphs and calculated as described in the statistical analyses section. Isotype controls include mouse (Ms), hamster (Hm), and rat (Rt) IgGs (gray bars).

Fig. 5. LPS stimulates expression and secretion of IL-6 and GM-CSF, and secretion of MIP1 α from lupus prone primary MCs. Primary MRL/lpr MCs were cultured for 6 hrs in the absence (Control) or presence of increasing amounts of LPS. IL-6 (A), GM-CSF (B), and MIP1 α (C) were measured by individual ELISAs in the media. Primary MRL/lpr MCs were cultured for the indicated time in the absence (Control) or presence of 5 ng/ml LPS. IL-6 (D), GM-CSF (E), and MIP1 α (F) were measured by individual ELISAs in the media. *Il-6* (G), *Gm-csf* (H), and *Neut* (I) mRNA expression were measured by real-time RTPCR. Experiments were performed three times in A-C and twice in D-H. Global p-values (A-C) or individual p-values (D-I) are provided on the graphs. P-values were calculated across replicate experiments as described in the statistical analyses section with representative experiments presented.

Fig. 6. Inhibiting NEU activity or TLR4 significantly reduces LPS-stimulated secretion of IL-6, GM-CSF, and MIP1 α , and expression of *Il-6* and *Gm-csf*. Primary MRL/lpr MCs were cultured in the absence or presence of 500 μ M NEU inhibitor oseltamivir phosphate (OP) and then stimulated with 5 ng/ml LPS for 6 hrs. IL-6 (A), GM-CSF (B), and MIP1 α (C) were measured by individual ELISAs in the media. *Il-6* and *Gm-csf* (D) mRNA expression were measured by real-time RTPCR in the cells. E) Primary MCs were pre-treated without or with 500 μ M OP followed by stimulation with 5 ng/ml LPS for 3 hrs and NEU activity was

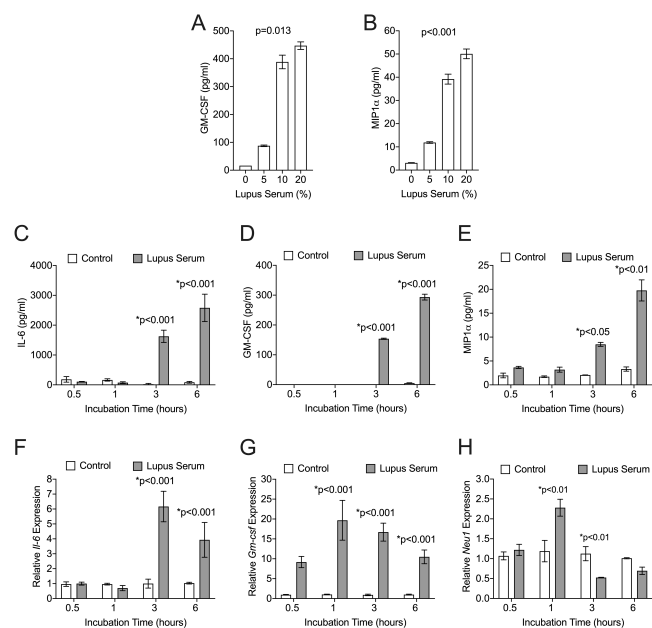
measured after addition of substrate to live cells. Control, treated with vehicle (water) only (no OP or LPS). F) Primary MCs were pre-treated without (No Ab) or with TLR4/MD2 antibodies and stimulated with 5 ng/ml LPS for 6 hrs. IL-6, GM-CSF, and MIP1 α were measured in the media by ELISA. Rt IgG, rat isotype control. Secretion in presence of LPS with No Ab was set to 1 for each cytokine and relative fold change in presence of TLR4/MD2 Ab calculated. Experiments in A, B, and E were performed three times and all other experiments were performed twice. P-values were calculated across replicate experiments as described in the statistical analyses section and provided on graphs of data from representative experiments.

Fig. 7. NEU activity mediates IL-6 and GM-CSF secretion through ERK or p38 MAPK signaling. A-B) MRL/lpr primary MCs were incubated without inhibitor (Control) or with 10 μ M ERK (U0126), p38 (SB203580), JNK (SP600125), or PI3K (Wortmannin) inhibitors for 2 hrs prior to addition of 10% lupus serum (A) or 5 ng/ml LPS (B) for 3 hrs. Data for one experiment representative of three independent experiments is presented. Levels of all three cytokines across replicate experiments were similar to levels observed in Figs. 1-2 in response to lupus serum and in Fig. 5 in response to LPS. P-values were calculated across replicate experiments as described in the statistical analyses section. #, significant increase at $p < 0.001$ vs Control. All other p-values are provided on the graphs. Graphs are data from representative experiments. C-D) Cells were pre-treated with vehicle (water) or 500 μ M NEU inhibitor oseltamivir phosphate (OP) for 16 hrs and then stimulated with 10% lupus serum (C) or 5 ng/ml LPS (D) for 15-45 min. Whole cell extracts were prepared and subjected to western immunoblot using antibodies to phosphorylated ERK or p38 (Phospho), or total ERK or p38 (Total). Ratio of band intensities for phosphorylated to Total for ERK and p38 are provided below each blot. Results are representative of three independent experiments with consistent decreases observed after stimulation for 45 min with Lupus Serum (C) and 30 min with LPS (D) in the presence of OP vs absence of OP.

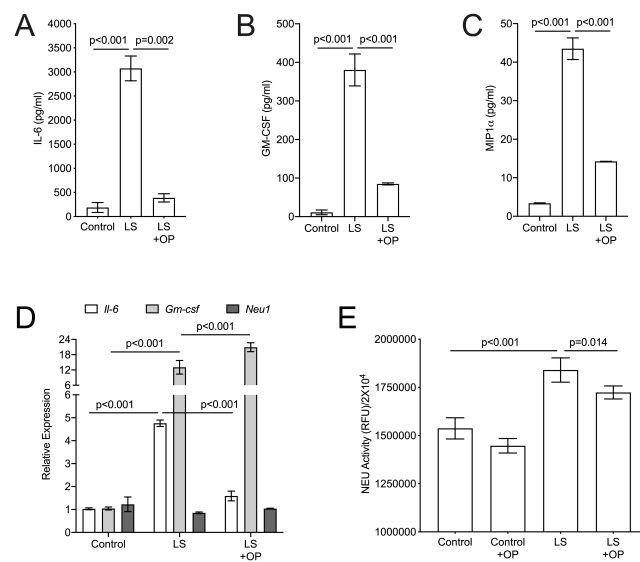
Fig. 8. Blocking NEU activity significantly increased sialic acid-containing N-linked glycoproteins in lupus serum-stimulated MCs. MRL/lpr Primary MCs were pre-treated with Vehicle (water) or with 500 μ M NEU activity inhibitor oseltamivir phosphate (OP) and stimulated with 10% lupus serum (LS) for 3 hrs. N-glycans were analyzed by MALDI-FTICR. Intensities of 19 sialylated N-glycans clearly identified after background subtraction are presented. Structures for sialylated N-glycans for which the structures are known, including m/z 2122 and m/z 2853 that were significantly increased by OP, are shown on the graph. Data

presented are the average of replicate wells +SD. Adjusted p-values on the graph were calculated as described in Materials and Methods.

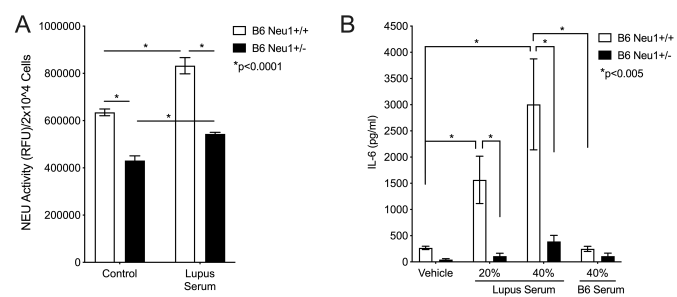
Fig. S1. OP is not toxic to MCs. MRL/lpr Primary MCs were pre-treated with Vehicle (water) or with 500 μ M NEU activity inhibitor oseltamivir phosphate (OP) and stimulated with 10% lupus serum (LS) or 5 ng/ml LPS for 3 hrs. Cell viability was determined by the MTT assay. Data is the mean +SD of 4 replicates and presented as percent proliferation relative to unstimulated (Unstim) cells treated with vehicle, which was set to 100%. MCs treated with 0.1 saponin served as a control.



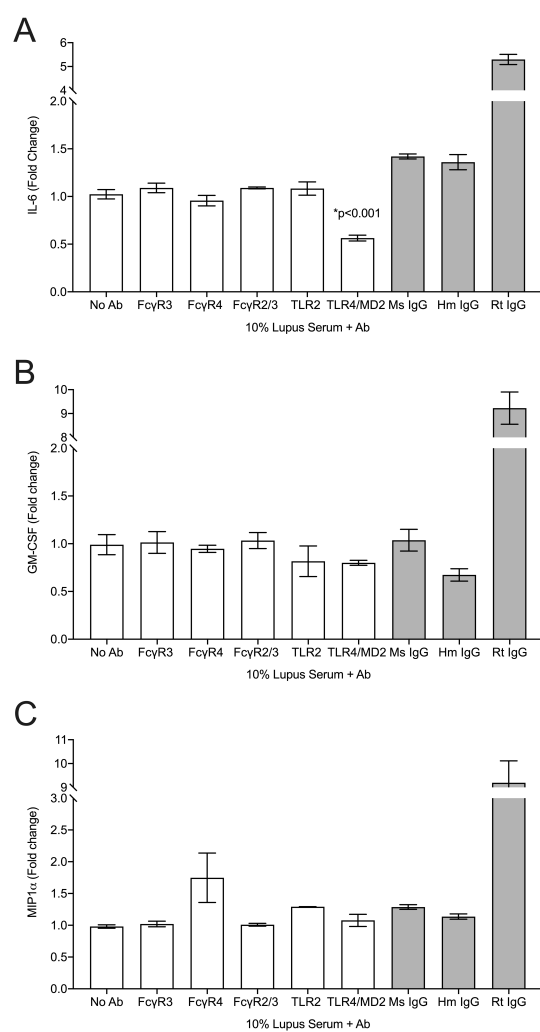
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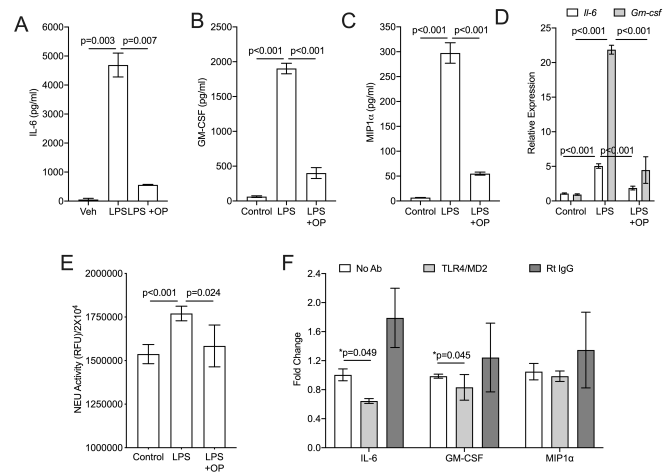
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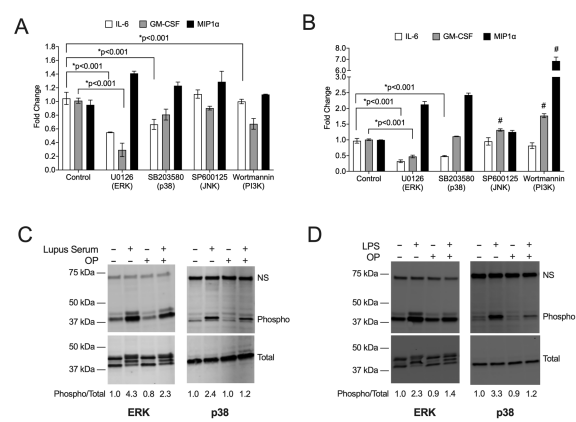
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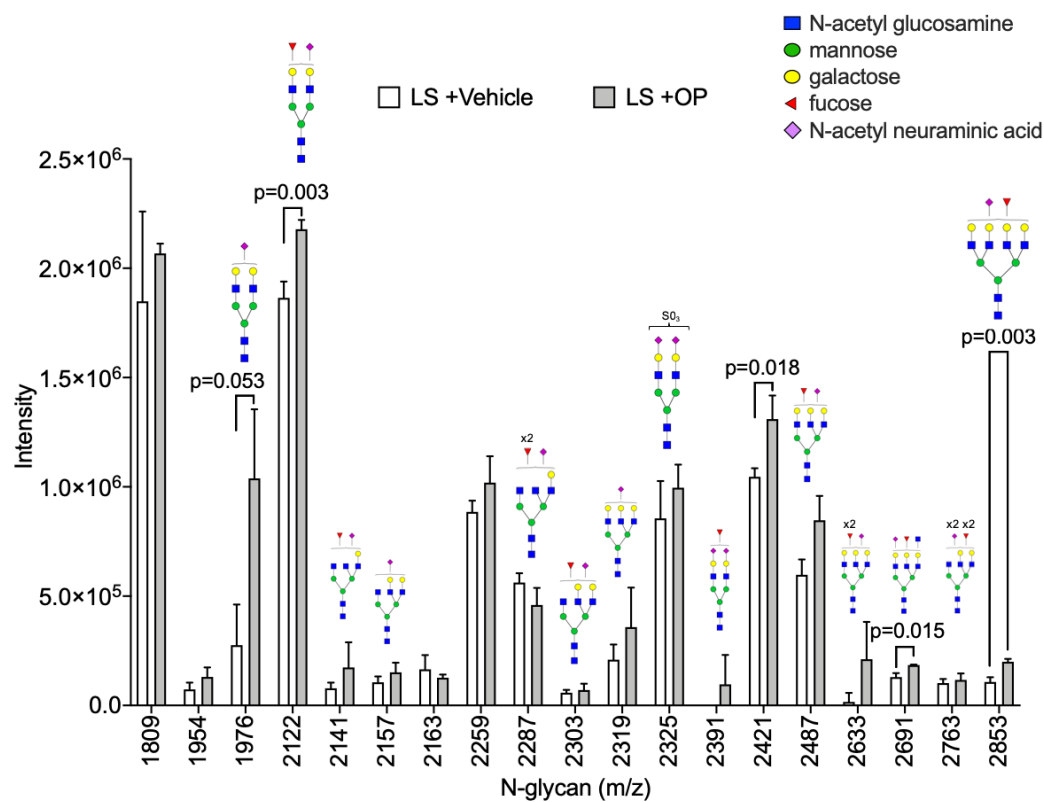
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The role of neuraminidase 1 (NEU1) in cytokine release by primary mouse mesangial cells and disease outcomes in murine lupus nephritis.

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The role of neuraminidase 1 (NEU1) in cytokine release by primary mouse mesangial cells and disease outcomes in murine lupus nephritis.

Running title: *NEU1 in mesangial cell function and lupus nephritis*

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Abstract

The importance of altered glycosphingolipid (GSL) metabolism is increasingly gaining attention as a characteristic of multiple chronic kidney diseases. Previously, we reported elevated levels of glycosphingolipids and neuraminidase (NEU) enzyme activity/expression in the urine or kidney of lupus patients and lupus prone mice, and demonstrated NEU activity mediates the production of cytokines by lupus prone mouse primary mesangial cells. This mediation occurs in part through TLR4 and p38/ERK MAPK signaling in response to LPS and lupus serum. However, the precise role of NEU1, the most abundant NEU in the kidney, is incompletely known. In this study we investigated the effect of genetically reduced *Neu1* levels *in vitro* and *in vivo*. Mesangial cells from non-autoimmune prone *Neu1*^{+/-} C57BL/6 mice had significantly reduced NEU activity, cytokine expression, and cytokine secretion in response to lupus serum and LPS, thereby suggesting reducing *Neu1* expression may reduce the inflammatory response in lupus nephritis. Disease was assessed in female B6.SLE1/2/3 lupus prone mice with genetically reduced levels (*Neu1*^{+/-}) or wild-type levels (*Neu1*^{+/+}) of *Neu1* from 28 to 44 weeks of age along with aged matched C57BL/6 controls. Renal disease was unexpectedly mild in all B6.SLE1/2/3 mice despite evidence of systemic disease. B6.SLE1/2/3 *Neu1*^{+/-} mice exhibited significantly reduced levels of renal NEU1 expression and changes in renal α -2,6 linked sialylated N-glycans compared to the *Neu1*^{+/+} or healthy C57BL/6 mice, but measures of renal and systemic disease were similar between the B6.SLE1/2/3 *Neu1*^{+/+} and *Neu1*^{+/-} mice. We conclude that NEU1 is the NEU largely responsible for mediating cytokine release by mesangial cells, at least *in vitro*, but may not be involved in modulating renal glycosphingolipid levels *in vivo* or impact onset of nephritis in lupus prone mice. The impact of the altered renal sialylated N-glycan levels and potential role of NEU1 with respect to established nephritis (late disease) in lupus prone mice bears further investigation.

Keywords: neuraminidase, lupus, IL-6, mesangial cells, sialylation, glycosphingolipid

Introduction

Systemic lupus erythematosus (SLE) is a chronic inflammatory autoimmune disorder that affects multiple organs, including the kidney. Glomerulonephritis (GN) affects up to 70% of lupus patients and is a leading cause of morbidity and mortality. Lupus nephritis (LN), along with other kidney diseases such as polycystic kidney disease and diabetic nephropathy, is characterized by altered glycosphingolipid (GSL) metabolism. Levels of glucosylceramides (GlcCers) [or hexosylceramides (HexCers), which are GlcCers + galactosylceramides], lactosylceramides (LacCers), and neuraminidase (NEU) activity are elevated in the urine or kidney of lupus patients and mice with nephritis compared to controls [1,2]. The precise role GSL metabolism plays in the kidney and LN is not completely understood. To this end, our laboratory analyzed the role of NEU activity in the function of mesangial cells, cellular early responders to immune complex deposition in LN. NEU is an enzyme that removes terminal sialic acids from glycolipids in the GSL catabolic pathway and from glycoproteins. Of the four mammalian NEUs described, NEU1 is more highly expressed in the kidney followed by NEU3. Expression of *Neu3* is approximately 40-fold lower than *Neu1* at the mRNA level [2-8].

We previously demonstrated NEU activity mediates the production of IL-6, GM-CSF, and MIP1 α by primary kidney MCs from lupus prone MRL/lpr mice in response to lupus serum and occurs in part through TLR4 and p38/ERK MAPK signaling [2,9]. In particular, IL-6, which plays an important role in the onset of nephritis [10-15], seems to be directly regulated by NEU-mediated TLR4-MAPK signaling. Blocking NEU activity with the viral NEU inhibitor oseltamivir phosphate (OP) significantly inhibited mammalian NEU activity and cytokine release in primary MRL/lpr MCs *in vitro* [2,16] despite also significantly increasing the levels of LacCers and HexCers [16]. OP treatment of MRL/lpr mice at the onset of proteinuria did not improve disease measures and actually resulted in a further slight elevation of GSLs [16]. These results suggest the continued/further elevation of renal GSLs may play a more important role in driving renal disease than reducing NEU activity. Alternatively, the two NEUs most highly expressed in the kidney (NEU1 and NEU3) may have differential effects in MCs or on disease that

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3 may not be appreciated by pharmacologically inhibiting all NEU activity. Thus, we used a genetic
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5 knockdown of the *Neu1* gene to determine the role of NEU1 in MCs *in vitro* and on lupus disease
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7 development *in vivo*.
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10 A genetic knockdown of *Neu1* in the non-autoimmune prone C57BL/6 strain (B6 *Neu1*^{+/-}) was obtained. Primary
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12 MCs were isolated from kidneys of B6 *Neu1*^{+/-} and *Neu1*^{+/+} littermates to determine if specifically reducing *Neu1*
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14 expression mediates cytokine release as demonstrated when inhibiting all NEU activity with OP [2,9]. The *Neu1*
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16 knockout was crossed onto the lupus prone B6.SLE1/2/3 strain from the B6 *Neu1*^{+/-} strain to generate B6.SLE1/2/3
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18 *Neu1*^{+/-} mice to determine if altering NEU1 levels impacts disease development. We demonstrate that primary B6
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20 *Neu1*^{+/-} MCs exhibited significantly decreased NEU activity and secreted significantly less IL-6 and GM-CSF
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22 compared to wild-type MCs (B6 *Neu1*^{+/+}) in response to lupus serum and LPS. These results suggest NEU1 plays
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24 an integral role in mediating cytokine release from MCs. B6.SLE1/2/3 *Neu1*^{+/+} mice had significantly decreased
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26 renal levels of sialylated α 2,6-linked N-glycans compared to healthy C57BL/6 controls. These levels were partially
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28 rescued in the B6.SLE1/2/3 *Neu1*^{+/-} mice. Despite these observed *in vitro* and *in vivo* effects, genetically reducing
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30 *Neu1* expression in the B6.SLE1/2/3 lupus prone mouse strain did not significantly impact renal or systemic disease
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32 onset.
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42 **Materials and methods**

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44 *Ethics statement and mouse strains*

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47 All animal studies and methods of euthanasia were approved by the local Institutional Animal Care and Use
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49 Committee (IACUC) and by the US Army Medical Research and Materiel Command (USAMRMC) Animal Care
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51 and Use Review Office (ACURO). Breeding pairs of B6.SLE1/2/3 were purchased (Stock #007228, Jackson
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53 Laboratory, Bar Harbor, ME) and bred in-house. Breeding pairs of C57BL/6J mice
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3 were purchased (Stock #000664, Jackson Laboratory, Bar Harbor, ME) and bred in-house. Breeding pairs
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5 of C57BL/6 *Neu1*^{+/-} mice were generously provided by Dr. Alessandra d'Azzo from St. Jude's
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7 Children's Research Hospital and bred in-house. C57BL/6 *Neu1*^{+/-} mice were bred with the B6.SLE1/2/3
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9 mice to generate B6.SLE1/2/3 *Neu1*^{+/-} mice through speed congenics. Mice were maintained on a 12 h
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11 light-dark cycle with access to food (PicoLab 5V5M) and water ad libitum. Breeding pairs of MRL/lpr
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13 mice were purchased (Stock #000485, Jackson Laboratory, Bar Harbor, ME) and bred in-house for blood
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15 collections. Blood from 3-4 MRL/lpr or C57BL/6 female or male mice at 16-18 weeks of age were pooled
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17 by strain and sex, and serum collected. Each pooled serum collection was tested for endotoxin using
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19 Pierce Chromogenic Endotoxin Quantification Kit (Thermo Scientific, Rockford, IL) and only serum
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21 collections with undetectable levels of endotoxin were used for experiments.
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27 ***Generation and culturing of primary mesangial cell lines***

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30 Kidney glomeruli were isolated from 6-week-old female C57BL/6 (B6) *Neu1*^{+/+} and B6 *Neu1*^{+/-} female
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32 mice for generation of two independent primary mesangial cell lines (MCs). The generation and
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34 characterization of primary MCs were previously described [2,9]. All cell lines were regularly tested for
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36 mycoplasma (Applied StemCell, Inc, Milpitas, CA) and genotype was confirmed by PCR as described
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38 below. Cells were grown in a 5% CO₂ atmosphere at 37°C in low glucose MEM/D-valine medium with
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40 20% fetal bovine serum, 1% penicillin/streptomycin and ITS supplement (5 µg/ml insulin, 5 µg/ml
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42 transferrin, 5 ng/ml sodium selenite) (GenDEPOT, Houston, TX; Sigma-Aldrich, St. Louis, MO) and cell
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44 passages 6-9 were utilized for all experiments. Cells were serum starved prior to stimulation with 5-20%
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46 serum from MRL/lpr (lupus serum) or 5-45 ng/ml ultra-pure lipopolysaccharide (LPS, Invivogen, San
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48 Diego, CA) for 6 hrs as indicated in the figure legends.
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55 ***Generation of B6.SLE1/2/3 *Neu1*^{+/-} mice and genetic study***

B6 *Neu1*^{+/-} and B6.SLE1/2/3 mice were bred to generate B6.SLE1/2/3 *Neu1*^{+/-} mice and backcrossed for five generations before being used in the study. Tail DNA was extracted at weaning and used to genotype the mice for *Neu1* and the three NZM2410 lupus susceptibility loci *Sle1*, *Sle2*, and *Sle3*. PCR for *Neu1* was performed following a protocol supplied by the lab of Dr. Alessandra d’Azzo using multiplex primers MNTG-1, MNTG-2, and LacZ to detect the wild-type allele (MNTG-1 and MNTG-2), and knockout allele (MNTG-1 and LacZ). PCR for the three congenic SLE (*Sle1*, *Sle2*, and *Sle3*) intervals was performed using primers for simple-sequence repeat markers essentially as reported previously [17-20]. Primer sequences and expected PCR amplicon sizes for each genotype is provided in Table 1. Prior to being used in the genetic study, mice were confirmed to have NZM2410-derived genotype for the three SLE loci at nine out of the eleven SLE susceptibility alleles and C57BL/6J at D7MIT40 as expected. D7MIT30 and D7MIT31 at the proximal end of chromosome 7 were reported to be NZM2410-derived [18,19]. However, our genotyping results indicated that all B6.SLE1/2/3 mice, including the breeding pairs purchased from Jackson Laboratories were homozygous for the C57BL/6J strain. DNA from NZM2410 mice and C57BL/6J DNA were used as controls. Tan coat color and pink eye color were also used as phenotypic indicators of homozygous NZM2410 genotype at Chromosome 4 *b* loci and Chromosome 7 *p* loci, respectively in the B6.SLE1/2/3 mice.

B6.SLE1/2/3 *Neu1*^{+/+} and B6.SLE1/2/3 *Neu1*^{+/-} female littermates (14 per group) were monitored bi-weekly for onset of proteinuria using Chemstrip 7 protein strips (Roche, Indianapolis, IN). Five female C57BL/6 were used as age-matched controls. Twenty-four-hour (24 h) urine and serum were collected every two weeks beginning at 28 weeks of age to 44 weeks of age (study endpoint). Mice were euthanized and organs immediately harvested for analyses at the study endpoint. Body weight, spleen weight, and spleen length were recorded at the study endpoint. One kidney was flash frozen and one kidney was fixed in 10% buffered formalin phosphate (Fisher Scientific, Waltham, MA) and paraffin embedded. Approximately one-fourth of the spleen was used for flow cytometry and the rest was flash frozen and stored.

MTT (tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay

Primary MCs (10,000 cells per well) were grown in CoStar 3603 black 96-well clear-bottom plates and stimulated with 10% MRL/lpr lupus serum or 5 ng/ml LPS for 24, 48 and 72 hrs. After incubation, percentage of cells proliferating in response to stimulant compared to vehicle at each time point was measured using a Cell-Quanti-MTT Cell Viability Assay Kit according to manufacturer's instructions (BioAssay Systems, Hayward, CA) and calculated as $[\text{Abs}(\text{Treatment}) - \text{Abs}(\text{Treatment blank})] / [\text{Abs}(\text{Vehicle}) - \text{Abs}(\text{Vehicle blank})] * 100\%$ at each time point.

ELISA analyses

Commercially available ELISA kits were used to measure IL-6 (BioLegend, San Diego, CA) and GM-CSF (R&D systems, Minneapolis, MN) in primary MC culture medium according to the manufacturer's instructions. Cytokine levels were normalized to total protein concentration of cells. Total protein was measured using a Micro BCA Protein Assay Kit (Thermo Scientific, Rockford, IL). Final concentrations of cytokines were adjusted to an equivalent of 25 μg of protein for comparison across experiments.

Real-time PCR

Total RNA was extracted from primary MCs or kidney cortices using the RNeasy kit (Qiagen, Hilden, Germany) and cDNA was reverse transcribed with 0.5–1 μg RNA using the iScript cDNA synthesis kit (BioRad, Hercules, CA). Message levels of *Il-6*, *Gm-csf*, or *β -actin* were measured by real-time PCR as described [9].

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Live cell neuraminidase activity assay

NEU activity in live primary MCs (20,000 cells per well) was measured essentially as described previously [2,9] using 15 μ M substrate 2' (4-methylumbelliferyl)- α -D-N-acetylneuraminic acid (4MU-NANA) in TBS (Sigma Aldrich; St. Louis, MO). Fluorescence intensity was measured using a SpectraMax i3c fluorometer with SoftMax Pro 6 software (Molecular Devices, San Hose, CA) prior to (baseline) and at the indicated time intervals after substrate addition. Relative fluorescence units (RFU) per 1000 cells is presented on the graphs.

Neuraminidase activity assay and lipid measures in kidney homogenates

Frozen kidney cortex tissue was homogenized in 20 mM Tris HCl pH 7.4 buffer containing protease inhibitors (Roche, Indianapolis, IN) as described previously [1]. Protein concentration was measured using the BCA assay. NEU activity of kidney homogenates (100-250 μ g) was measured using Amplex Red Neuraminidase Assay Kit according to manufacturers' protocol (Invitrogen A22178; Carlsbad, CA). Levels of hexosylceramides (HexCers) and lactosylceramides (LacCers) in kidney homogenates were quantified by the Medical University of South Carolina Lipidomics Core Facility as previously described [1,2,16].

Urine Albumin ELISA

As described previously, urine was collected over a 24 h period using individual metabolic cages and total albumin levels measured by ELISA [16]. Concentrations were calculated using OD values that fell within the linear portion of the standard curve and averaged for each sample.

Renal pathology assessment

Fixed kidney sections from each of the study mice were stained with H&E or PAS and evaluated by a pathologist blinded to the strain/genotype and scored as described previously [16]. Presented scores are the total for all features.

Serum IgG ELISA and flow cytometry for activated T cells

Total IgG levels in mouse sera were measured by ELISA as described previously [16]. Concentrations were calculated using OD values that fell within the linear portion of the standard curve and averaged for each sample. Activated T cells were measured using approximately one-quarter of each spleen as described previously [16].

Immunohistochemistry of renal sections

Kidneys were fixed in 10% buffered formalin phosphate (Fisher Scientific, Waltham, MA), paraffin embedded, and sectioned (3-4 μ M). Sections were deparaffinized, endogenous peroxidase activity blocked, and permeablized with 0.5% Triton X-100. After blocking with 5% BSA, sections were incubated with rabbit anti-NEU1 or anti-NEU3 (Santa Cruz Biotechnology Inc., Santa Cruz, CA), or the following biotinylated lectins; 30 μ g/ml Maackia amurensis lectin II (MAL II), 10 μ g/ml Maackia amurensis Lectin I (MAL I), or 10 μ g/ml Sambucus nigra lectin (SNA) (Vector Laboratories, Burlingame, CA). Sections were then incubated with HRP conjugated goat anti-rabbit secondary antibody (Invitrogen, Carlsbad, CA) for NEU1 and NEU3, or Avidin-HRP conjugate (BioLegend, San Diego, CA) for the lectins. DAB substrate solution (Vector Laboratories, Burlingame, CA) was used to detect HRP and sections counterstained in Carazzi's Hematoxylin. Staining with secondaries alone were used as negative controls. Images of random fields were captured on a Nikon Eclipse 90i microscope with a Nikon Digital Sight DS-Fi1 camera and NIS-Elements AR 5.1 software. Staining intensity was measured using ImageJ software [21,22] as described previously [2]. Mean and standard deviations of staining intensity for entire fields (3

random fields per mouse) or individual glomeruli (present in the 3 random fields) are presented for 3-8 mice as indicated in figure legends.

Statistics

Association between treatment or duration of exposure to lupus serum or LPS with outcomes (e.g. protein, mRNA levels, GSLs, NEU activity) in the B6 *Neu1*^{+/+} compared to the *Neu1*^{+/-} MCs were evaluated by two-way ANOVA and adjusted for multiple comparison by Tukey correction where appropriate. All *in vitro* experiments were performed at least twice with similar results as indicated in the figure legends. For the mouse study, global differences between B6, B6.SLE1/2/3 *Neu1*^{+/+}, and B6.SLE1/2/3 *Neu1*^{+/+} mice for different outcomes measured at 44 weeks were evaluated using the global Type III F-test from linear models including type as a main effect or the Kruskal Wallace test if linear model assumptions were not met. If global significance was found at $p < 0.05$, pair-wise comparisons were conducted between groups using Tukey's HSD to adjust for multiple comparisons. Associations between mouse genotype with albumin and IgG over time were evaluated using a linear mixed model (LMM) approach. Models included main effects for mouse genotype and time, and a random mouse effect to account for correlation between measures collected on the same animal. Interaction between mouse genotype and time was also considered but excluded from the model if statistical significance was not met. For LMM, model assumptions were checked graphically and transformations were considered if necessary. Correlation between HexCer16 with renal pathology score or with urine albumin at 44 weeks in *Neu1*^{+/+} and *Neu1*^{+/-} was examined using Pearson's correlation. All analyses were conducted in SAS v 9.4 (SAS Institute, Cary NC). Adjusted p-values for pairwise comparisons are reported on the graphs and global p-values are provided in the text of respective results sections unless otherwise indicated in the figure legends.

Results

Reduced NEU activity in heterozygote NEU1 non-lupus prone C57BL/6 mesangial cells

Our previous results suggest NEU1 may be responsible for the observed effects in cytokine release by MCs. To determine if NEU1 is responsible for the observed NEU-mediated cytokine release from MCs [2,9], MCs from C57BL/6 (B6) *Neu1*^{+/-} mice were analyzed. B6 *Neu1*^{+/-} mice were previously shown to have ~50% reduced levels of *Neu1* message and a non-significant slight reduction of renal NEU activity [23]. We observed that the B6 *Neu1*^{+/-} mice in our colony also showed a slight, but not significant, reduction in overall NEU activity in kidney homogenates (Fig. 1A). We previously reported that primary MCs from B6 *Neu1*^{+/-} mice had significantly reduced NEU activity compared to MCs from B6 *Neu1*^{+/+} in a live-cell NEU activity assay at both baseline and following stimulation with lupus serum (LS) [9]. We further demonstrated B6 *Neu1*^{+/+} MCs released significantly more IL-6 in response to 20 or 40% LS compared to B6 *Neu1*^{+/-} MCs, and that neither *Neu1*^{+/+} or *Neu1*^{+/-} B6 MCs released IL-6 in response to 40% B6 serum [9]. Here we extend that observation to show that B6 *Neu1*^{+/+} MCs respond in a dose-dependent manner to 5%-20% LS by releasing significant amounts of IL-6 (Fig. 1B) and GM-CSF (Fig. 1C). B6 *Neu1*^{+/-} MCs do not release significant amounts of either cytokine. Similarly, a significant LS dose-dependent increase in mRNA expression was observed for both *Il-6* (Fig. 1D) and *Gm-csf* (Fig. 1E) in the B6 *Neu1*^{+/+} MCs and not in *Neu1*^{+/-} MCs. These results demonstrate that as little as 5% LS elicits a pathological response by MCs from non-autoimmune prone mice. Although reducing *Neu1* expression does not significantly reduce global renal NEU activity *in vivo*, it does decrease NEU activity and cytokine release by primary MCs *in vitro*.

NEU1 mediates LPS-stimulated cytokine release in B6 MCs

NEU activity mediates IL-6 and GM-CSF secretion in lupus prone MRL/lpr MCs in part through the TLR4 pathway [9]. To determine if the NEU activity that mediates this effect is attributed to NEU1, B6

Neu1^{+/+} and *Neu1*^{+/-} MCs were stimulated with the TLR4 ligand LPS. LPS elicited a significant increase in NEU activity in live B6 *Neu1*^{+/+} MCs (B6 *Neu1*^{+/+} Vehicle vs LPS, Fig. 2A), but not in live B6 *Neu1*^{+/-} MCs (B6 *Neu1*^{+/-} Vehicle vs LPS, Fig. 2A). NEU activity was significantly higher in the B6 *Neu1*^{+/+} MCs compared to the B6 *Neu1*^{+/-} MCs at both baseline (vehicle treated) and following stimulation with LPS (Fig. 2A). LPS significantly increased IL-6 secretion from both B6 *Neu1*^{+/+} MCs and B6 *Neu1*^{+/-} MCs with maximal secretion observed at the lowest dose (5 ng/ml) (Fig. 2B). The level of IL-6 secreted by the B6 *Neu1*^{+/+} MCs was significantly greater than that observed for the B6 *Neu1*^{+/-} MCs. Significant levels of GM-CSF were also secreted by B6 *Neu1*^{+/+} MCs, but not by B6 *Neu1*^{+/-} MCs, in response to LPS (Fig. 2C). LPS stimulated significant increases in *Il-6* (Fig. 2D) and *Gm-csf* (Fig. 2E) expression at the message level in the *Neu1*^{+/+} MCs and was maximal at the lowest dose of LPS for both genes. Although slight increases in *Il-6* (Fig. 2D) and *Gm-csf* (Fig. 2E) mRNA were observed in *Neu1*^{+/-} MCs, the increases were not statistically significant. These results suggest NEU1 mediates cytokine release of IL-6 and GM-CSF through a TLR4 signaling pathway that results in upregulation at the transcriptional level in non-autoimmune B6 MCs.

NEUs catabolize glycoproteins and glycolipids by removing terminal sialic acids. Accumulation of sialic acid glycolipids such as gangliosides can lead to cell-cycle arrest and hypertrophy in MCs [24,25]. An MTT assay was used to determine if significant reductions in IL-6 and GM-CSF secretion in stimulated B6 *Neu1*^{+/-} MCs could be attributed to differences in cell viability. Stimulation with LS or LPS elicited proliferation in both B6 *Neu1*^{+/+} and B6 *Neu1*^{+/-} MCs (Fig. 2F). The B6 *Neu1*^{+/-} MCs elicited similar percentages of proliferation as B6 *Neu1*^{+/+} + MCs at all time points in response to LS or LPS indicating a reduction of *Neu1* expression (and NEU activity) in B6 *Neu1*^{+/-} MCs does not significantly impact cell viability over 72 hrs. Together, the results in primary B6 MCs indicate the role of NEU activity in mediating cytokine release from MCs is largely due to NEU1, and suggest that reducing *Neu1* expression in lupus prone mice may reduce renal inflammation and improve disease.

Neu1 heterozygote knockout reduced renal NEU1 levels, but did not impact renal NEU activity or glycosphingolipid levels in B6.SLE1/2/3 lupus prone mice

To determine the effects of specifically reducing NEU1 on lupus disease development, we crossed the *Neu1* knockout onto the B6.SLE1/2/3 lupus prone strain. A homozygous knockout (*Neu1*^{-/-}) eliminated most NEU activity in the kidney suggesting NEU1 accounts for most renal NEU activity; however, the *Neu1*^{-/-} mice exhibited a renal phenotype [23]. Thus, we generated heterozygotes for this study to avoid confounding results. B6.SLE1/2/3 mice were derived from and develop disease similar to the NZM2410 lupus prone strain [17], which is characterized by increased renal NEU activity in nephritic mice [2], as observed in MRL/lpr lupus prone mice [26]. B6.SLE1/2/3 *Neu1*^{+/+} and *Neu1*^{+/-} littermates, and B6 healthy control mice were aged to 44 weeks of age. IHC of kidney sections confirmed NEU1 levels in both the overall tissue (total field) or specifically within the glomeruli were significantly reduced in *Neu1*^{+/-} compared to *Neu1*^{+/+} B6.SLE1/2/3 and compared to (*Neu1*^{+/+}) healthy control B6 mice (Fig. 3A). Renal NEU3 levels were not significantly different between the *Neu1*^{+/+} and *Neu1*^{+/-} B6.SLE1/2/3 mice (Fig. S1), indicating that NEU3 protein levels do not increase to compensate for the loss of NEU1 protein in the *Neu1*^{+/-} mice. The study describing the generation of the *Neu1* knockout reported NEU activity was non-significantly reduced approximately 30% in *Neu1*^{+/-} compared to *Neu1*^{+/+} kidneys [23]. Our NEU assay on homogenates from kidney cortices of 44 week-old B6.SLE1/2/3 *Neu1*^{+/-} mice showed only a slight, insignificant decrease in NEU activity (Fig. 3B), similar to what was observed in the B6 strain in Fig. 1. Renal NEU activity was expected to be increased in the B6.SLE1/2/3 mice at what should be late stage disease. However, NEU activity was not different in the B6.SLE1/2/3 mice compared to the B6 healthy control mice. We then measured effects on renal GSL metabolism. Renal LacCers and HexCers are increased in both MRL/lpr [1,2] and NZM2410 (Fig. S2) mice in late disease. In both the *Neu1*^{+/+} and *Neu1*^{+/-} B6.SLE1/2/3 mice, none of the renal LacCer chain lengths (Fig. 3C) and only two of the major HexCer chain lengths (C16 and C24:1) (Fig. 3D; global p-values of 0.012 and 0.011, respectively) were increased compared to the B6 healthy controls. There were no significant differences

in the levels of any of the chain lengths or overall total LacCers or HexCers between *Neu1*^{+/+} and *Neu1*^{+/-} B6.SLE1/2/3 mice. The similar levels of renal NEU activity, LacCers, and HexCers, in the B6.SLE1/2/3 mice suggest an unexpectedly mild renal disease at 44 weeks of age, and reducing NEU1 levels did not have a significant effect on renal GSL metabolism.

Effect of reducing NEU1 expression on renal sialylated glycan levels

NEU1 is thought to have greater affinity for sialylated glycoproteins than for sialylated glycolipids (gangliosides). Since reducing NEU1 levels did not appear to have an effect on renal glycosphingolipid levels, we stained renal sections with lectins MAL II, MAL I, and SNA, which preferentially recognize α -2,3 linked sialic acids on O-glycans, α -2,3 linked sialic acids on N-glycans, and α -2,6 linked sialic acids on N-glycans, respectively. MAL II staining was observed almost exclusively in glomeruli, particularly in endothelial cells and podocytes. MAL I showed strong staining of proximal tubules and weaker staining in glomerular endothelial cells, while SNA stained glomeruli, as well as some tubules (Fig. 4, representative IHC images). Staining intensity was quantified in entire image fields or within glomeruli only and presented graphically below the IHC images in Fig. 4. Staining for MAL II was significantly increased in both the *Neu1*^{+/+} or *Neu1*^{+/-} B6.SLE1/2/3 mice compared to the B6 mice across the entire section (global p-value< 0.001). This was likely due to observed increased staining outside the glomeruli and was not impacted by reduced levels of NEU1. Conversely, staining for SNA was significantly higher in B6 compared to either *Neu1*^{+/+} or *Neu1*^{+/-} B6.SLE1/2/3 in the glomeruli (global p-value= $\leq$ 0.001) where most staining is present and suggests increased desialylation of N-glycans with α 2,6 linked sialic acid in the lupus kidney. SNA staining was increased in the glomeruli of *Neu1*^{+/-} compared to the *Neu1*^{+/+} B6.SLE1/2/3; however, the difference did not reach statistical significance. MAL I staining, which was observed to be more global than MAL II or SNA staining, was also compared between the *Neu1*^{+/+} and *Neu1*^{+/-} B6.SLE1/2/3 kidneys. A small, yet significant, increase in MAL I staining across

the tissue was observed in the *Neu1*^{+/-} sections, but was not observed for the staining in the glomeruli.

These results suggest that α -2,6 linked sialic acids on N-glycans are reduced in lupus prone mice developing nephritis, and a heterozygote knockout of *Neu1* may partially rescue sialylation of these glycans.

Knocking down Neu1 in B6.SLE1/2/3 lupus prone mice did not improve disease

Mice were monitored for onset of proteinuria by measuring albumin levels in 24 hr urine samples collected every two weeks from 28 to 44 weeks of age. Endpoint urine albuminuria (Fig. 5A) was not significantly different between the *Neu1*^{+/+} and *Neu1*^{+/-} mice, and change in urine albuminuria from 28 to 44 weeks was slightly, but not significantly, lower in the *Neu1*^{+/-} mice (Fig. 5B). However, albuminuria was greater than 30 μ g/mouse/24 hrs in only two of the *Neu1*^{+/+} mice and four of the *Neu1*^{+/-} mice, and was only slightly elevated compared to B6 mice by the 44-week endpoint of the study (global p-value= 0.042). There also were no significant differences in renal pathology scores between the B6.SLE1/2/3 *Neu1*^{+/+} and *Neu1*^{+/-} mice and minimal renal disease pathology observed for most B6.SLE1/2/3 mice compared to B6 healthy control mice (Fig. 5C). Although NEU1 mediates *Il-6* expression and IL-6 release by MCs *in vitro*, renal expression of *Il-6* was essentially undetectable in most of the B6.SLE1/2/3 mice with amplification observed at >32 cycles, which was similar to that for the healthy B6 mice (Fig. 5D). Animals that presented with high albuminuria also had high renal scores; however, there was no correlation between albuminuria and *Il-6* expression, or between renal scores and *Il-6* expression. Interestingly, renal HexCer C16 levels significantly correlated with both urine albumin (Fig. 5E) and renal pathology scores (Fig. 5F), suggesting that the B6.SLE1/2/3 lupus prone strain exhibits increased renal glycosphingolipids as disease progresses, similar to the MRL/lpr and NZM2410 strains. Together, these data indicate the B6.SLE1/2/3 mice had only mild renal disease and suggest that genetically reducing renal NEU1 levels may not delay onset of nephritis.

NEU activity and GSL metabolism are known to be important for immune cell function, specifically with regard to lupus [27,28]. Although the B6.SLE1/2/3 mice only had mild nephritis at 44 weeks of age, they were exhibiting hallmarks of systemic disease. Total spleen size (Fig. 6A, global p-value= 0.001) and spleen to body weight ratio (Fig. 6B, global p-value< 0.001) were significantly increased in both the *Neu1*^{+/+} and *Neu1*^{+/-} B6.SLE1/2/3 mice compared to B6 mice, and similar between the *Neu1*^{+/+} and *Neu1*^{+/-} groups. Serum IgG levels at the end point of the study were significantly elevated in both the *Neu1*^{+/+} and *Neu1*^{+/-} B6.SLE1/2/3 mice compared to the B6 mice (Fig. 6C, global p-value< 0.001), but no significant difference was observed between the *Neu1*^{+/+} and *Neu1*^{+/-} B6.SLE1/2/3 mice. The serum IgG levels steadily increased at similar rates, as well as having similar levels, over the study period in the *Neu1*^{+/+} and *Neu1*^{+/-} B6.SLE1/2/3 mice (Fig. 6D, global p-value< 0.001). Since NEU1 was also shown to play a role in T cell activation [29,30], activated T cells were analyzed by flow cytometry. The percentage of activated T cells in the spleens of B6.SLE1/2/3 *Neu1*^{+/-} were similar to those in the *Neu1*^{+/+} B6.SLE1/2/3 mice (Fig. 6E). Together, these results indicate B6.SLE1/2/3 mice were developing systemic disease and reducing NEU1 expression did not delay the onset or progression.

Discussion

Mounting evidence indicates GSL metabolism is altered in LN and may play a role in disease progression, specifically with respect to the function of MCs and immune cells [1,2,16,26,28]. Our results treating primary lupus prone MCs with the NEU activity inhibitor oseltamivir phosphate (OP) demonstrated blocking NEU activity inhibits release of several cytokines including IL-6 and GM-CSF, but results in further accumulation of GSLs [2,9,16]. We demonstrated that one mechanism by which NEU activity mediates cytokine release in MCs is through TLR4, a sialylated glycoprotein[9]. Our results here show that knocking out one allele of the *Neu1* gene significantly reduced NEU activity, and cytokine

expression or release of IL-6 and GM-CSF from MCs of non-lupus prone mice in response to lupus serum and LPS. These results indicate NEU1 is largely responsible for the NEU activity mediating TLR4 signaling of IL-6 release from MCs, which may be an important inflammatory mechanism in non-lupus prone as well as lupus prone MCs. Both TLR4 and IL-6 are implicated as pivotal players in the progression of lupus and nephritis in particular [31,32], and MCs were shown to be the major cell type producing IL-6 in LN [33]. Our results are consistent with results demonstrating a role for NEU1 in signal transduction in other cell types [30,34-40] with a direct role in the desialylation of TLR4 in macrophages, microglia, and 3T3-adipocyte cells [34,35,38].

To determine if blocking NEU1 specifically would impact renal GSL metabolism and disease in lupus nephritis, we generated a genetic knockout of *Neu1* on the lupus prone B6.SLE1/2/3 strain. We chose the B6.SLE1/2/3 strain because it contains three lupus susceptibility loci from the NZM2410 lupus prone strain, which we showed exhibits increased NEU activity as disease progresses [2], was reported to develop disease similar to the NZM2410 strain [17], and is on the B6 background allowing for use of the B6 mice as controls and potential comparison to *in vitro* results with the B6 MCs. Since a homozygous knockout of *Neu1* on the C57BL/6 background results in a renal phenotype and splenomegaly [23], we generated a heterozygote knockout to avoid confounding results.

Surprisingly, the B6.SLE1/2/3

(*Neu1*^{+/+}) mice, both purchased from Jackson Laboratories and bred in our facility, developed disease more slowly than previously reported [17]. Approximately 50% of B6.SLE1/2/3 mice were expected to succumb to disease by 32-36 weeks of age. In our study, only one mouse of each B6.SLE1/2/3 genotype group (*Neu1*^{+/+} and *Neu1*^{+/-}) succumbed to disease before 36 weeks of age. Thus, we extended the study to 44 weeks. Despite significantly reduced renal NEU1 protein levels, significant effects on renal disease as assessed by urine albumin, pathological scoring, and *Il-6* message levels were not observed in *Neu1*^{+/-} compared to the *Neu1*^{+/+} mice. However, as mentioned above, these measures showed that most B6.SLE1/2/3 *Neu1*^{+/+} mice had only mild disease when compared to the B6 healthy controls. This

was further confirmed when analyzing renal NEU activity and GSL levels, which were anticipated to be increased in the lupus prone mice developing nephritis but were similar to the levels in healthy B6 mice. Importantly, the association of renal HexCer C16 with urine albumin and renal pathology scores in the B6.SLE1/2/3 mice support that increasing levels of renal GSLs is a phenomenon common across lupus prone mouse strains and may serve as a general biomarker of LN.

We also did not observe significant differences in systemic disease development including splenomegaly, serum IgG levels, and activated T cell numbers. The lack of an effect on disease could have been due to the non-significant reduction in global renal NEU activity. Renal NEU activity in both the B6.SLE1/2/3 and B6 strains was not significantly different between *Neu1*^{+/+} and *Neu1*^{+/-} when analyzing kidney homogenates. This was not likely due to compensation by increasing NEU3 protein levels. One explanation may be a feedback loop by which NEU1 activity is increased in response to reduced NEU1 expression as a means of maintaining activity levels. Additional support for a potential NEU feedback mechanism includes our previous *in vitro* results in MRL/lpr MCs that demonstrated NEU activity increases in response to lupus serum stimulation at the same time *Neu1* mRNA levels are reduced [9]. Thus, the renal NEU activity levels maintained in the *Neu1*^{+/-} mice could be due to increased activity of NEU1 or NEU3, which the assay does not definitively distinguish between. Alternatively, *Neu1* knockdown in microglia was shown to reduce only surface NEU activity [35]. Since the activity assay used in kidney homogenates measured global NEU activity and not just cell surface activity, we were unable to determine if the genetic reduction of NEU1 (*Neu1*^{+/-}) impacted cell surface renal NEU activity specifically. However, our live cell activity assays *in vitro* comparing *Neu*^{+/+} and *Neu1*^{+/-} B6 MCs suggest genetic reduction of *Neu1* significantly reduced cell surface NEU activity.

Pharmacological treatment of MRL/lpr mice with a NEU inhibitor resulted in slightly further elevating renal LacCers, HexCers, and GM3 *in vivo* and significantly increasing these GSLs in MCs *in vitro* [16]. Our results here indicate reducing NEU1 levels does not impact renal GSL levels *in vivo*. Again, this could be due to a non-significant change in NEU activity. Alternatively, NEU1 was shown to have greater specificity for sialylated glycoproteins while NEU3 showed preference for sialylated glycolipids such as gangliosides [41-45]. Thus, we performed lectin staining to more globally examine sialylated glycoprotein levels. We observed significantly decreased glomerular SNA staining in B6.SLE1/2/3 lupus prone mice compared to non-autoimmune prone B6 mice, consistent with observations in human renal biopsies from lupus nephritis patients [46]. The B6.1/2/3 *Neu1*^{+/-} mice showed a trend towards increased glomerular SNA staining compared to *Neu1*^{+/+} B6.SLE1/2/3 mice. SNA was shown to bind TLR4 indicating it can be sialylated via an α 2,6 linkage and removal of the α 2,6 linked sialic acid by NEU1 was required for LPS-mediated signaling [47]. Together our *in vivo* and *in vitro* results suggest increasing renal NEU activity decreases glomerular α 2,6-linked sialylated glycans as disease progresses, and reducing NEU1 levels may restore (increase) the levels of these sialylated glycans that may include TLR4. Our collective *in vitro* studies in MCs here and previously [2,9] suggest NEU1 may modulate TLR4 and other sialylated α 2,6 linked glycans in the kidney during disease progression. However, reducing renal NEU1 levels did not impact disease onset, or disease measures at least at the mild disease stage analyzed. The significance of altered sialylated glycan levels remains to be determined. Identifying which glycans are altered and determining their function may provide a better understanding of disease development.

The development of only mild renal disease in the B6.SLE1/2/3 mice was an important limitation of the *in vivo* study. The mice were followed to 44 weeks of age, at which point approximately 90% were expected to have succumbed to disease as reported previously [17]. The B6.SLE1/2/3 mice were clearly developing systemic disease based on measures of splenomegaly and serum IgG levels. However, spleen

size observed in our mouse colony averaged ~300 mg at 44 weeks of age whereas spleen weight was previously reported to average ~550 mg by 28 weeks of age [17]. The mild disease development was not likely a result of drift in our colony as analyses of mice directly from the vendor similarly developed mild disease. This could have been due to environmental differences. Diet was shown to modulate the genetic susceptibility of nephritis in the lupus prone strain NZM2410. A Western diet resulted in 90% of NZM2410 mice developing nephritis while mice on a control diet resulted in only 40% of mice developing nephritis [48]. Mice in our colony were provided chow similar in crude fat content as the Western diet, but was lower in cholesterol, butter fat, and sugar content similar to the control diet. Thus, diet differences could have accounted for the mild disease development by 44 weeks in our study mice. Alternatively, detailed genotyping of our B6.SLE1/2/3 mouse colony, including breeders obtained from Jackson Labs used to establish our colony, indicated the proximal end of loci SLE3 was B6-derived instead of NZM2410-derived as expected [18,19]. It is unknown if this difference potentially influences disease onset/development.

In conclusion, our results demonstrate that NEU1 is largely responsible for the NEU-mediated IL-6 and GM-CSF release through a TLR4 signaling pathway in primary MCs, which can be activated by serum from lupus mice. Despite the mild disease development, HexCer C16 levels correlated with urine albumin and renal pathology scores in B6.SLE1/2/3 mice and strongly suggest that altered renal GSL metabolism is a common feature in lupus prone mouse strains. Reducing renal NEU1 levels *in vivo* did not impact disease onset. However, we demonstrated significantly altered glomerular α -2,6 linked sialylated N-glycans in the B6.SLE1/2/3 lupus strain and these levels may be modulated by NEU1 activity as disease progresses. Our results here combined with results in several mouse strains and human patient samples [1,2,9,16,46] suggest renal GSLs and sialylated glycans may potentially serve as biomarkers of disease. Further studies to define the role and mechanisms of GSL metabolism, sialylated glycans, and NEU activity in LN is warranted.

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Table 1. Primers used for genotyping mice. F, forward; R, reverse

Locus	Primer	Sequence	Product Size	
			<i>Neu1</i> ^{+/+}	<i>Neu1</i> ^{+/-}
<i>Neu1</i>	MNTG-1	5'- GAC AGG GAT CGC CGG GAG CTA TGG-3'	180 bp	180 bp & 400 bp
	MNTG-2	5'-CAC CAG GCT GAA GTC ATC CTC TGC-3'		
	LacZ	5'- GAT AGG TCA CGT TGG TGT AGA TGG		
		GCG-3'		
			B6	NZM
<i>Sle1</i>	D1MIT15-F	5'-TCC ACA GAA CTG TCC CTC AA-3'	160 bp	180 bp
	D1MIT15-R	5'-ATA CAC TCA CAC CAC CCC GT-3'		
	D1MIT47-F	5'-CTG ACC TCC ACA CGA CCC-3'	191 bp	200 bp
	D1MIT47-R	5'-GCT TGG GAA ACT GGA TGA AA-3'		
	D1MIT113-F	5'-CCT CAA AAT CAG GAT TAA AAG GG-3'	200 bp	210 bp
	D1MIT113-R	5'-ACA TGG GGT GGA CTT GTG AT-3'		
	D1MIT155-F	5'-ATG CAT GCA TGC ACA CGT-3'	230 bp	200 bp
	D1MIT155-R	5'-ACC GTG AAA TGT TCA CCC AT-3'		
<i>Sle2</i>	D4MIT72-F	5'-CTT TGA CTG ACT GGG TGA TCT G-3'	195 bp	110 bp
	D4MIT72-R	5'-AGC CTT TGT TAG GTT GGT TTA GC-3'		
	D4MIT274-F	5'-GCA CAG GCC TTG TAA TCC TT-3'	85 bp	90 bp
	D4MIT7274-R	5'-ATG ACA AGG AAA GTG TTT CCT AGG-3'		
	D4MIT329-F	5'-CAA GAA GAT GGG GAG TCA GTG-3'	95 bp	92 bp
	D4MIT329-R	5'-TGT GTG ATC AGC CTA CCA GG-3'		
<i>Sle3</i>	ckMM-F	5'-CCA GAC CAT CTG ATC CAG ATC-3'	110 bp	150 bp
	ckMM-R	5'-GGA GGT TGC AGT GAA TTC AAG-3'		
	D7MIT157-F	5'-CAG ACA ACC ACA TTG TCA CAT G-3'	150 bp	145 bp
	D7MIT157-R	5'-TGA TCT GTG ACT TTC AGA CTT GTG-3'		

Figure legends

Fig. 1. Genetic knockdown of the *Neu1* gene decreased NEU activity, and cytokine expression and

secretion by MCs in response to lupus serum. A) Homogenates from kidney cortices of 15-18 week-old C57BL/6 *Neu1*^{+/+} and *Neu1*^{+/-} mice were used to measure total NEU activity. Primary C57BL/6 *Neu1*^{+/+} and *Neu1*^{+/-} MCs were cultured in the absence (0) or presence of 5, 10 or 20% of LS for 6 hrs. IL-6 (B) and GM-CSF (C) levels in the media were quantified by ELISA and *Il-6* (D) and *Gm-csf* (E) mRNA levels in the cells were quantified by real-time PCR. Mean of replicate wells +SD are presented on the graphs. Graphs presented are representative of three experiments performed with independent lupus serum collections. Significant differences were determined as described in the methods and individual p-values after adjusting for multiple comparisons are provided on the graphs.

Fig. 2. Genetic knockdown of the *Neu1* gene decreased NEU activity, and cytokine expression and

secretion by MCs in response to LPS. A) Primary MCs from B6 *Neu1*^{+/+} and *Neu1*^{+/-} mice cultured in Vehicle or 5 ng/ml LPS for 3 hrs were used to measure NEU activity using a live cell assay. Activity was measured over time (5-30 min) after addition of NEU substrate to the cultures. Graph presented is representative of three experiments. Individual p-values comparing No serum to LS at each time point are indicated above each point for the *Neu1*^{+/+} or below each point for *Neu1*^{+/-} (*p<0.05; +p<0.01; ^p<0.001). Global p-values comparing activity in *Neu1*^{+/+} vs *Neu1*^{+/-} for Vehicle or LPS are provided to the right of the graph, #p<0.0001. Primary B6 *Neu1*^{+/+} and *Neu1*^{+/-} MCs were cultured with Vehicle or 5, 15 or 45 ng/ml LPS for 6 hrs. IL-6 (B) and GM-CSF (C) levels in the media were quantified by ELISA and *Il-6* (D) and *Gm-csf* (E) mRNA levels in the cells were quantified by real-time PCR. Mean of replicate wells +SD are presented on the graphs. Graphs presented are representative of three experiments. Significant differences were determined as described in the methods and individual p-values after adjusting for multiple comparisons are provided on the graphs. F) Percentage of proliferating cells were measured by MTT assay in primary B6 *Neu1*^{+/+} and *Neu1*^{+/-} MCs cultured in the absence or presence of 10% lupus serum (LS) or 5 ng/ml LPS for 24, 48, and 72 hrs. Percentages of proliferating

cells are relative to unstimulated cells. Graph presents mean $\pm$ SD and is representative of three independent experiments with similar results.

Fig. 3. Genetic knockdown of the *Neu1* gene in B6.SLE1/2/3 lupus prone mice reduced renal NEU1 levels, but did not alter levels of global NEU activity, LacCers, or HexCers in the kidney.

B6.SLE1/2/3 (SLE1/2/3) *Neu1*^{+/+} and *Neu1*^{+/-} mice, and C57BL/6 (B6) mice were aged to 44 weeks. A) Kidneys from 4 mice of each genotype were sectioned and stained for NEU1 expression. Quantification of staining intensity across 6 random fields per mouse (Total Field) or within all glomeruli of three random fields per mouse (Glomeruli) is presented graphically to the right of the representative stained sections. Data are presented as mean +SD. Kidney homogenates were used to measure total renal NEU activity (B), LacCer levels (C), and HexCer levels (D) for each mouse. Data are presented as mean +SD. Significant differences were determined as described in the methods. Adjusted p-values of pairwise comparisons are provided on the graphs.

Fig. 4. Genetic knockdown of *Neu1* partially rescues the significant decrease in renal N-glycans containing α -2,6 linked sialic acids in B6.SLE1/2/3 mice. Renal sections from the B6.SLE1/2/3 *Neu1*^{+/+} and *Neu1*^{+/-} mice, and C57BL/6 mice were stained with lectins to analyze relative levels of sialylated glycans. MAL II was used to stain renal sections from 5 *Neu1*^{+/+}, 6 *Neu1*^{+/-}, and 3 B6 mice. SNA was used to stain renal sections from 8 *Neu1*^{+/+}, 8 *Neu1*^{+/-}, and 3 C57BL/6 mice. MAL I was used to stain renal sections from 4 *Neu1*^{+/+} and 4 *Neu1*^{+/-} mice. Quantification of staining intensity across 6 random fields per mouse (Total Field) or within all glomeruli of three random fields per mouse (Glomeruli) is presented graphically below representative stained sections. Staining in multiple fields and glomeruli within those fields were quantified and data are presented as mean +SD. Significant differences were determined as described in the methods. Adjusted p-values of pairwise comparisons are provided on the graphs.

Fig. 5. Genetic knockdown of the *Neu1* gene in B6.SLE1/2/3 lupus prone mice did not delay or slow development of renal disease. Urine albumin was measured at two week intervals between 28 and 44 weeks of age for each mouse with endpoint levels (A) and change in levels from 28 to 44 weeks of age (B) are presented as a box plot with median +SD. Renal pathology was scored (C) and renal *Il-6* mRNA levels quantified (D) for each mouse at 44 weeks with data presented as mean +SD. Significant differences were determined as described in the methods. Adjusted p-values of pairwise comparisons are provided on the graphs. Correlations of renal HexCer chain length C16 with urine albumin levels at 44 weeks (E) or renal pathology scores (F) in all of the B6.SLE1/2/3 mice (both *Neu1*+/+ and *Neu1*+/-) were evaluated by Pearson's correlation. Two-tailed p-values (95% confidence interval) and r values between data sets are provided on the graphs.

Fig. 6. Genetic knockdown of the *Neu1* gene in B6.SLE1/2/3 lupus prone mice did not improve systemic disease measures. Systemic disease was analyzed in the B6.SLE1/2/3 (SLE1/2/3) *Neu1*+/+ and *Neu1*+/- mice, and C57BL/6 (B6) mice in Figs. 4 and 5. Splenomegaly was evaluated by measuring total spleen weight (A) and spleen to body weight ratio (B). Data are presented as mean +SD. Serum IgG levels were measured over time (28-44 weeks every other week). Endpoint serum IgG levels are presented as box plot with median (C) and levels over time are presented as mean +SD (D). Activated T cell numbers in spleen were quantified by flow cytometry using antibodies to CD3, CD44, and CD62L with percentage of activated (CD44+, CD62L-) CD3+ cells presented as mean +SD. Significant differences were determined as described in the methods. Adjusted p-values for pairwise comparisons are provided on the graphs.

Fig. S1. Renal LacCers are significantly elevated in NZM2410 mice as they age. LacCers levels were measured in kidney homogenates from 8-10 week-old or 23-28 week-old NZM2410 mice. Levels of the individual major LacCer chain lengths and the sum total of all LacCer chain lengths are presented.

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Significant difference between the 8-10 and 23-28 week-old mice was evaluated by ANOVA correcting for multiple comparisons by Sidak testing with the global p-value provided on the graph.

Fig. S2. Renal NEU3 levels are similar in the B6.SLE1/2/3 *Neu1*^{+/+} and *Neu1*^{+/-} mice. NEU3 levels were quantified in renal sections of B6.SLE1/2/3 (SLE1/2/3) *Neu1*^{+/+} and *Neu1*^{+/-} mice, and C57BL/6 (B6) mice at 44 weeks of age as in Fig. 3A. Staining intensities across 4 random fields per mouse are presented as mean $\pm$ SD. B6, n= 2 mice; B6.SLE1/2/3 *Neu1*^{+/+}, n= 2 mice; B6.SLE1/2/3 *Neu1*^{+/-}, n= 3 mice). Significant differences were determined as described in the methods. Adjusted p-values of pairwise comparisons are provided on the graph.

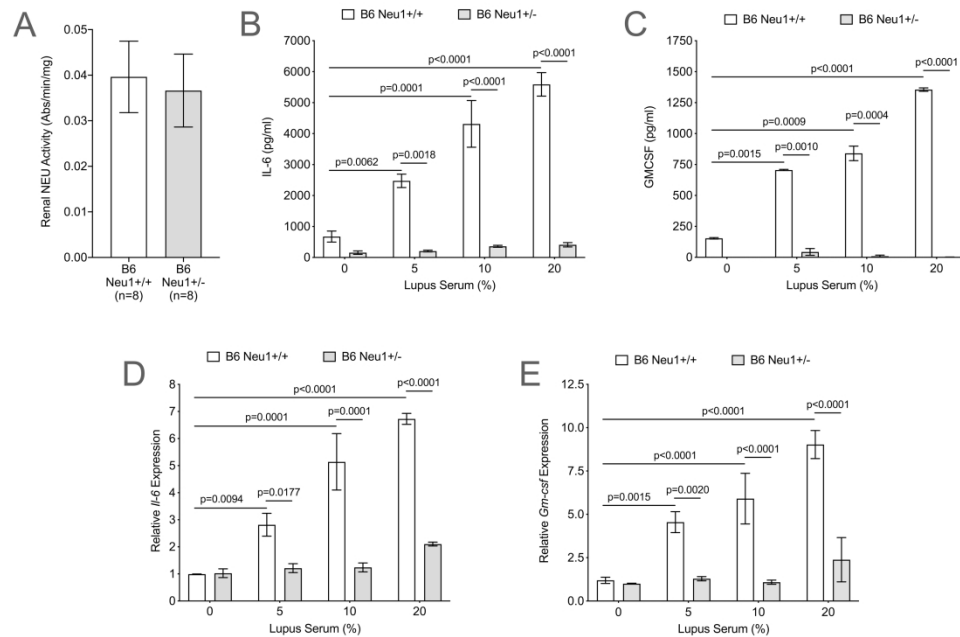


Fig. 1. Genetic knockdown of the Neu1 gene decreased NEU activity, and cytokine expression and secretion by MCs in response to lupus serum. A) Homogenates from kidney cortices of 15-18 week-old C57BL/6 Neu1+/+ and Neu1+/- mice were used to measure total NEU activity. Primary C57BL/6 Neu1+/+ and Neu1+/- MCs were cultured in the absence (0) or presence of 5, 10 or 20% of LS for 6 hrs. IL-6 (B) and GM-CSF (C) levels in the media were quantified by ELISA and IL-6 (D) and Gm-csf (E) mRNA levels in the cells were quantified by real-time PCR. Mean of replicate wells +SD are presented on the graphs. Graphs presented are representative of three experiments performed with independent lupus serum collections. Significant differences were determined as described in the methods and individual p-values after adjusting for multiple comparisons are provided on the graphs.

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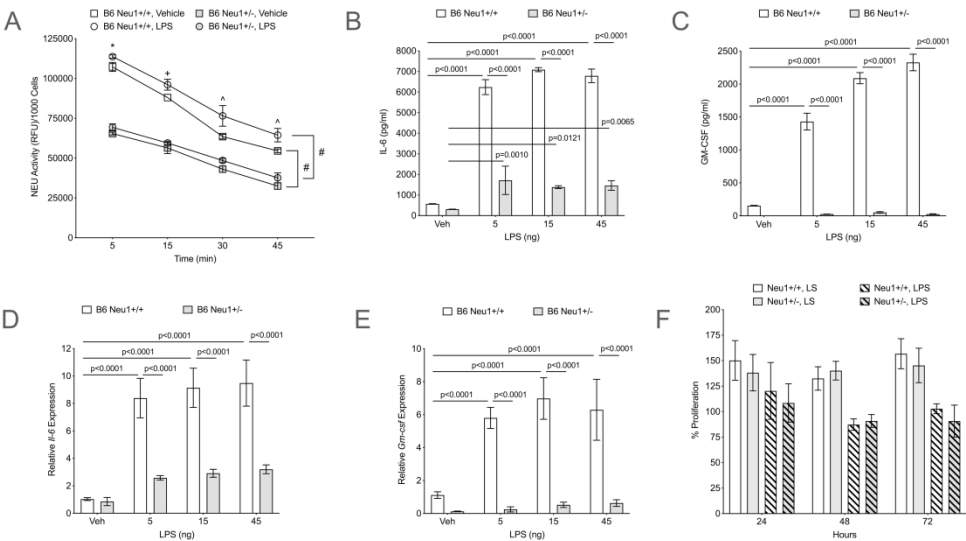


Fig. 2. Genetic knockdown of the Neu1 gene decreased NEU activity, and cytokine expression and secretion by MCs in response to LPS. A) Primary MCs from B6 Neu1+/+ and Neu1+/- mice cultured in Vehicle or 5 ng/ml LPS for 3 hrs were used to measure NEU activity using a live cell assay. Activity was measured over time (5-30 min) after addition of NEU substrate to the cultures. Graph presented is representative of three experiments. Individual p-values comparing No serum to LS at each time point are indicated above each point for the Neu1+/+ or below each point for Neu1+/- (*p<0.05; +p<0.01; ^p<0.001). Global p-values comparing activity in Neu1+/+ vs Neu1+/- for Vehicle or LPS are provided to the right of the graph, #p<0.0001. Primary B6 Neu1+/+ and Neu1+/- MCs were cultured with Vehicle or 5, 15 or 45 ng/ml LPS for 6 hrs. IL-6 (B) and GM-CSF (C) levels in the media were quantified by ELISA and Il-6 (D) and Gm-csf (E) mRNA levels in the cells were quantified by real-time PCR. Mean of replicate wells +SD are presented on the graphs. Graphs presented are representative of three experiments. Significant differences were determined as described in the methods and individual p-values after adjusting for multiple comparisons are provided on the graphs. F) Percentage of proliferating cells were measured by MTT assay in primary B6 Neu1+/+ and Neu1+/- MCs cultured in the absence or presence of 10% lupus serum (LS) or 5 ng/ml LPS for 24, 48, and 72 hrs. Percentages of proliferating cells are relative to unstimulated cells. Graph presents mean +SD and is representative of three independent experiments with similar results.

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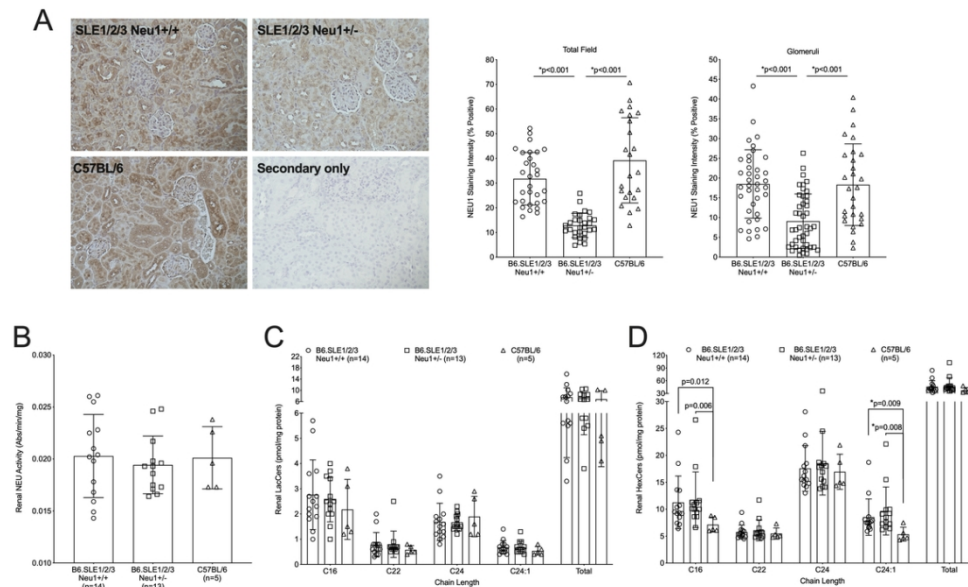


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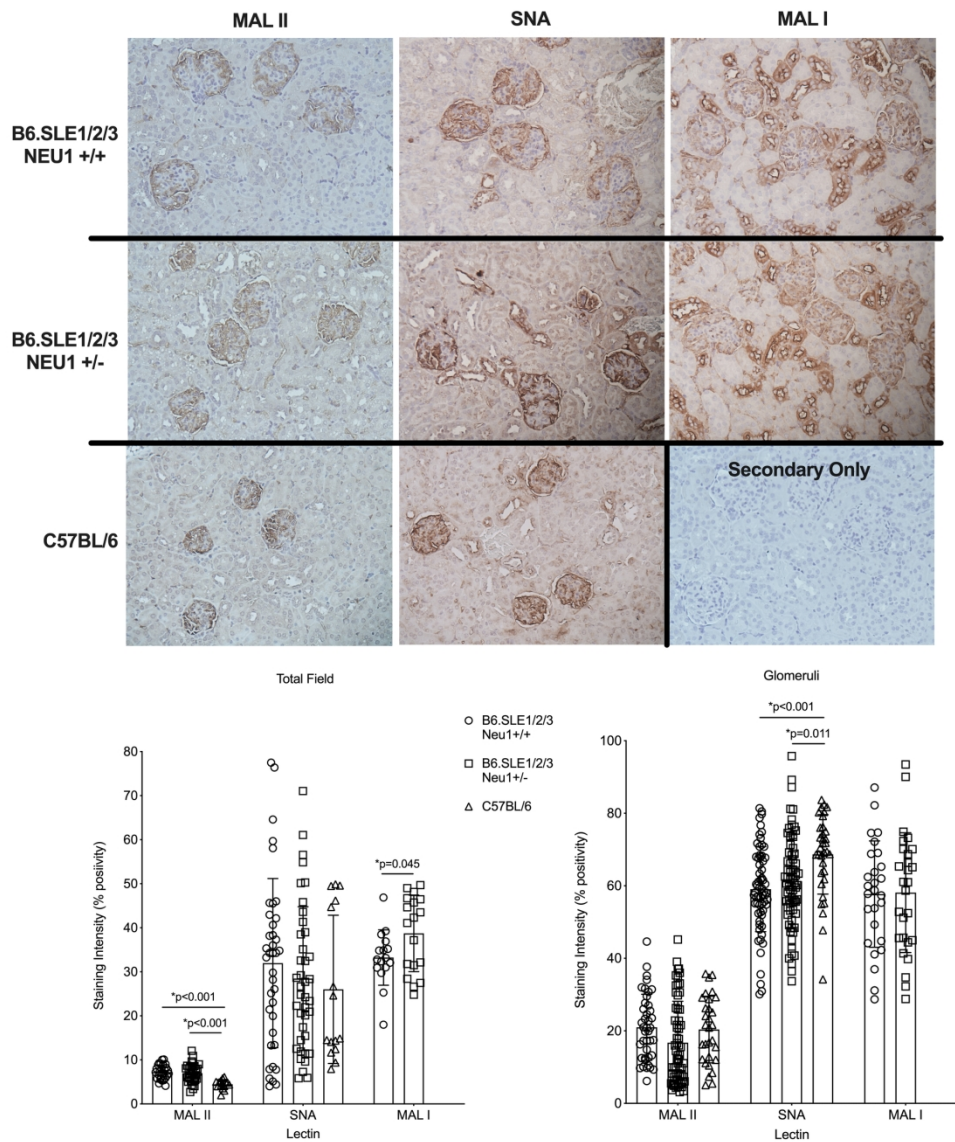


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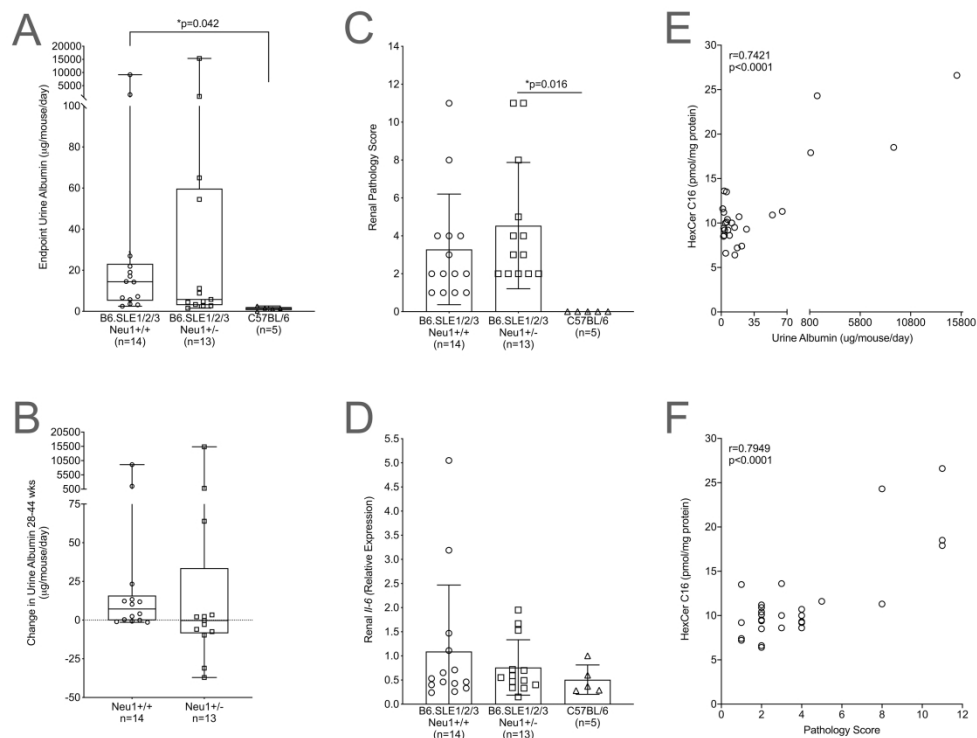


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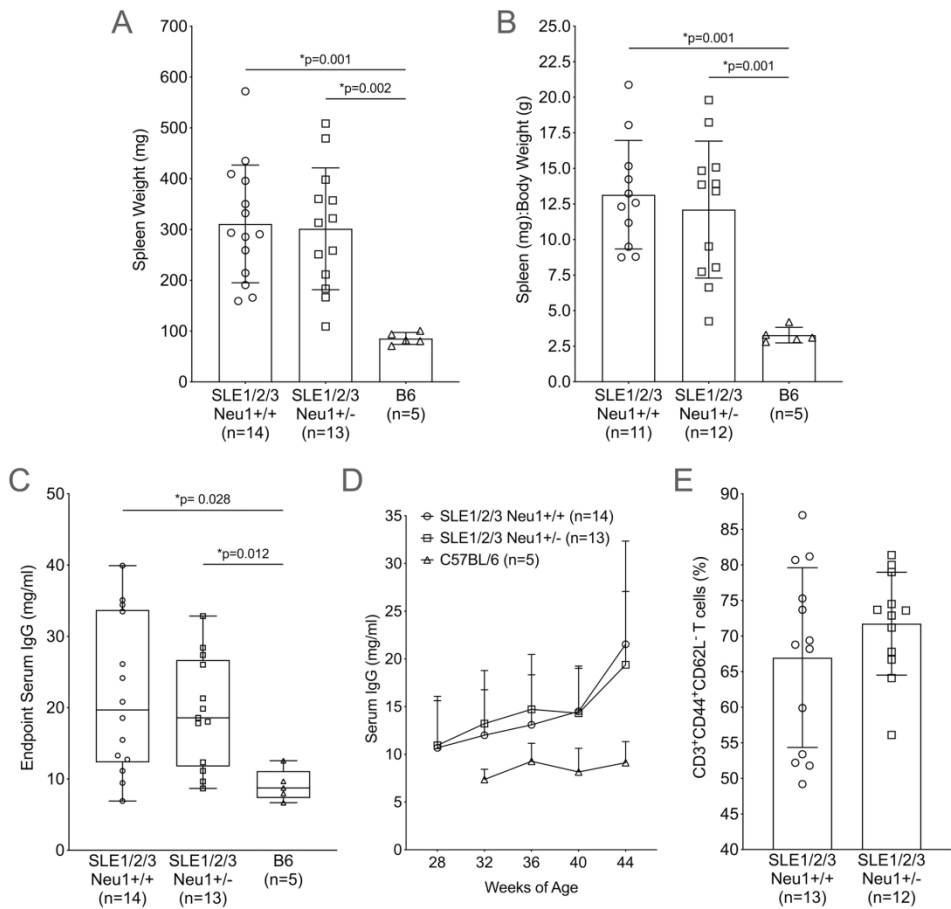


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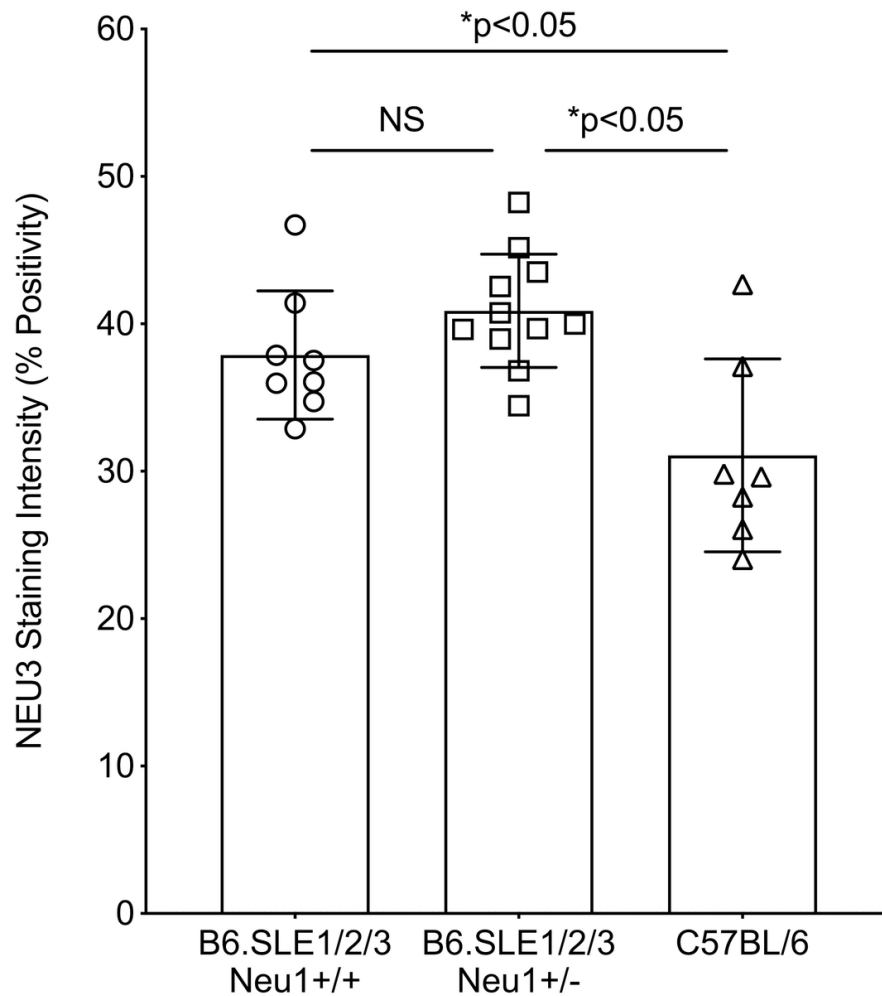


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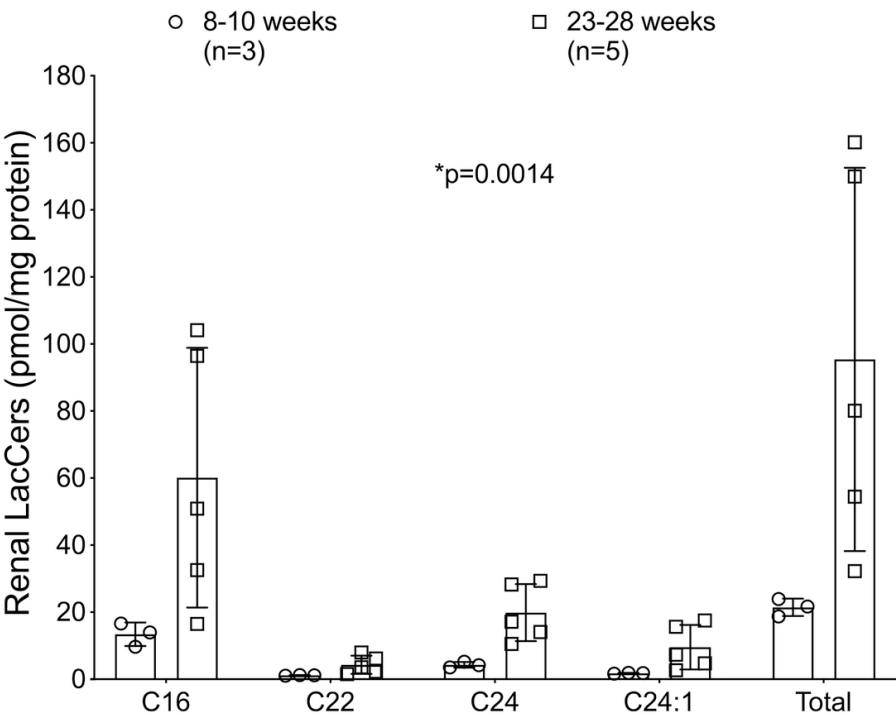


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