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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	4-10
4. Impact	10-11
5. Changes/Problems	12-13
6. Products	13-15
7. Participants & Other Collaborating Organizations	15-17
8. Special Reporting Requirements	17-18
9. Appendices	18-78

1. INTRODUCTION: *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

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: *Provide a brief list of keywords (limit to 20 words).*

Lupus nephritis, Glycosphingolipid, Biomarkers, Neuraminidase

3. ACCOMPLISHMENTS: *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

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. A manuscript describing the study drafted and submitted for review for publication in the journal PLOS One.

Major Task 2: Genetic knockout of Neu1 in lupus mice. The B6 Neu1 knockout has been 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. We are currently analyzing the data and drafting a manuscript for publication.

Major Task 3: GSLs as a predictor of response to therapy. Exosomes have been isolated from all 157 patient urine samples and lipid analyses completed. Validation of proteins identified in 10 discovery samples are being performed using individual ELISAs. Validated proteins will then be measured using these ELISAs in the remaining 157 exosome samples. This study is expected to be completed in the next 6 months of the no-cost extension period followed by manuscript preparation and publication during the remainder of the no-cost extension.

Major Task 4: Association of renal and urine GSLs with renal function. Sectioning of biopsy samples have been completed and MALDI-FTICR analyses on-going. We have encountered issues with both equipment malfunctions and tissue integrity with this study.

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

The major activities completed for the third year of the grant included: 1) completion of the OP treatment study in the MRL/lpr mice, including collection of samples, sample analyses and manuscript drafted and under review for publication; 2) completion of the genetic study including collection of samples and sample analyses data is being analyzed and a manuscript drafted; 3) exosome isolation from urine of the human patients and lipid identification completed, proteins identified in the 10 discovery sample proteomics are currently being validated; and 4) sectioning of renal biopsies have been completed and being analyzed by MALDI-FTICR. The specific objectives for this past year were to 1) complete mouse studies in both the MRL/lpr and B6.SLE1/2/3 mice and submit manuscripts for publication, 2) complete the exosome isolation and the proteomics and lipid discovery for the human urine exosomes sample analyses. All tasks were completed within the proposed timeline in the SOW except for the protein analyses of the patient exosomes samples and MALDI-FTICR analyses of the renal biopsies.

Major Task 1: Results for the MRL/lpr study are presented in the drafted manuscript 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 submitted for review and publication in the Journal of Biological Chemistry. A draft of that manuscript is 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: Data collected from analyses of samples from the B6.SLE1/2/3 Neu1+/+ vs Neu1+/- lupus mouse genetic study are nearing completion and a drafting of a manuscript is in the initial stages. Major findings from this study include:

- 1) Knocking out one allele of the GSL catabolic enzyme NEU1 (Neu1+/- mice) did not significantly impact overall renal NEU activity (Fig. 1A), 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. Thus, the renal levels of HexCers (Fig. 1B) and LacCers (Fig. 1C) also were not significantly altered in the Neu1+/- mice. Moreover, the lupus prone B6.SLE1/2/3 mice had only developed mild disease (see #2, Fig. 2 below), which is much later than previously reported for this lupus strain. Thus, renal NEU activity and HexCers/LacCers levels were unexpectedly similar to the age-matched healthy control C57BL/6 mice.

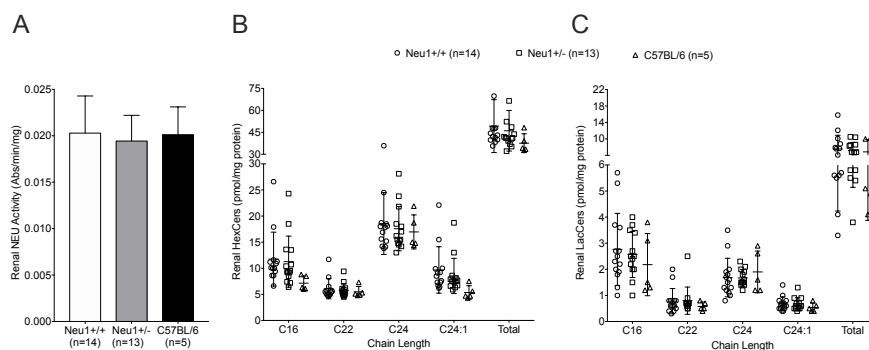


Fig. 1. Renal NEU activity and levels of HexCers and LacCers are similar in B6.SLE1/2/3 Neu1+/+ and Neu1+/- lupus mice. Kidneys from female 44 week-old B6.SLE1/2/3 Neu1+/+ and Neu1+/- lupus mice and healthy age-matched female C57BL/6 control mice were removed, homogenized, and analyzed for Neu activity using an activity assay (A), and for levels of GSLs HexCers (B), and LacCers (C) by SFC-MS/MS.

- 2) There were no significant differences in proteinuria (urine albumin) between the Neu1^{+/+} and Neu1^{+/-} B6.SLE1/2/3 mice, and proteinuria in most of the B6.SLE1/2/3 mice (both Neu1^{+/+} and Neu1^{+/-}) was mild compared to the non-lupus prone C57BL/6 healthy control mice (Fig. 2A-B). Renal pathology scoring is pending.

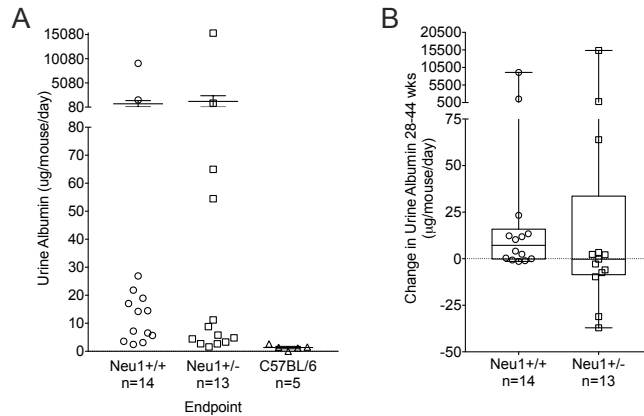


Fig. 2. Proteinuria is similar in B6.SLE1/2/3 Neu1^{+/+} and Neu1^{+/-} lupus mice. Urine was collected over a 24 hour period throughout the study and albumin levels quantified by ELISA as a measure of proteinuria. Urine albumin levels were compared between the B6.SLE1/2/3 Neu1^{+/+} and Neu1^{+/-} mice and healthy control C57BL/6 mice. A) Absolute urine albumin levels at the endpoint of the study (44 weeks of age). B) Change in urine albumin levels measure at 28 and 44 weeks of age.

- 3) Measures of systemic disease including splenomegaly (Fig. 3A-B) and serum IgG (Fig. 3C) were significantly higher in B6.SLE1/2/3 (both Neu1^{+/+} and Neu1^{+/-}) compared to healthy C57BL/6 control mice at the study endpoint (44 weeks of age), but were not significantly different in the B6.SLE1/2/3 Neu1^{+/+} compared to the Neu1^{+/-} mice. Activated T cell numbers (Fig. 3D) also were not different between the Neu1^{+/+} and Neu1^{+/-} mice.

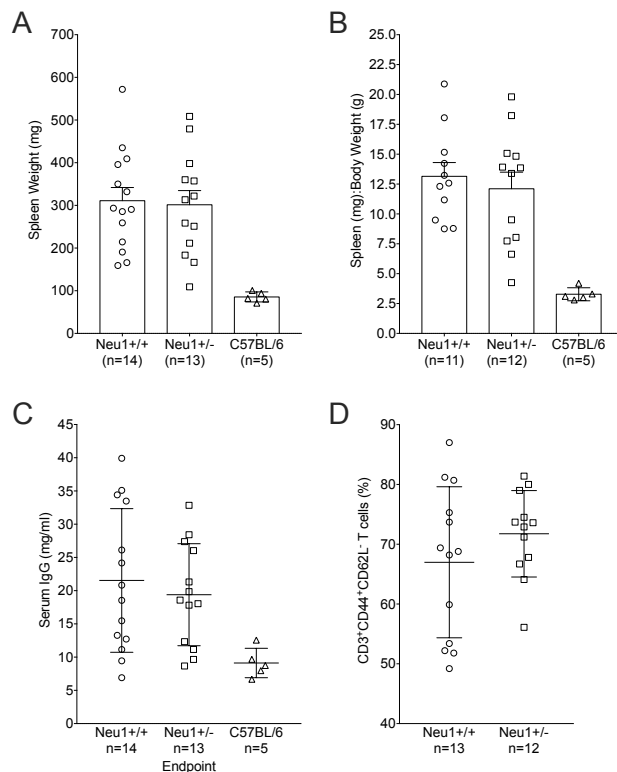


Fig. 3. Knocking out one allele of Neu1 does not impact systemic disease in B6.SLE1/2/3 lupus prone mice. Spleen weight (A) and spleen:body weight ratio (B), and serum IgG levels quantified by ELISA (C) in female 44 week-old B6.SLE1/2/3 lupus mice compared to healthy C57BL/6 mice. D) Numbers of activated T cells (CD3⁺ CD44⁺ CD62L⁻) were quantified by flow cytometry of dissociated spleen from the B6.SLE1/2/3 Neu1^{+/+} and Neu1^{+/-} mice.

4) We conclude reducing Neu1 levels in the B6.SLE1/2/3 lupus prone mice by knocking out one allele, does not significantly decreased NEU activity in the kidney and 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. Results from this genetic study are included in a manuscript being drafted that also includes results from experiments performed in vitro on primary mesangial cells (MCs) isolated from C57BL/6 Neu1+/+ and C57BL/6 Neu1+/- mice to determine how reducing Neu1 expression in these renal cells impacts their function. This data is presented below in #5.

5) Cultured primary MCs from Neu1+/+ and Neu1+/- C57BL/6 female mice were cultured in the absence or presence of lupus serum (Fig. 4) or LPS (Fig. 5). NEU activity (Fig. 4A), secretion of IL-6 (Fig. 4B) and GM-CSF (Fig. 4C), and mRNA expression of IL-6 (Fig. 4D) and GM-CSF (Fig. 4E) were all significantly lower in the Neu1+/- compared to the Neu1+/+ MCs stimulated with lupus serum. Similarly, NEU activity (Fig. 5A), secretion of IL-6 (Fig. 5B) and GM-CSF (Fig. 5C), and mRNA expression of IL-6 (Fig. 5D) and GM-CSF (Fig. 5E) were all significantly lower in the Neu1+/- compared to the Neu1+/+ MCs stimulated with TLR4 ligand LPS.

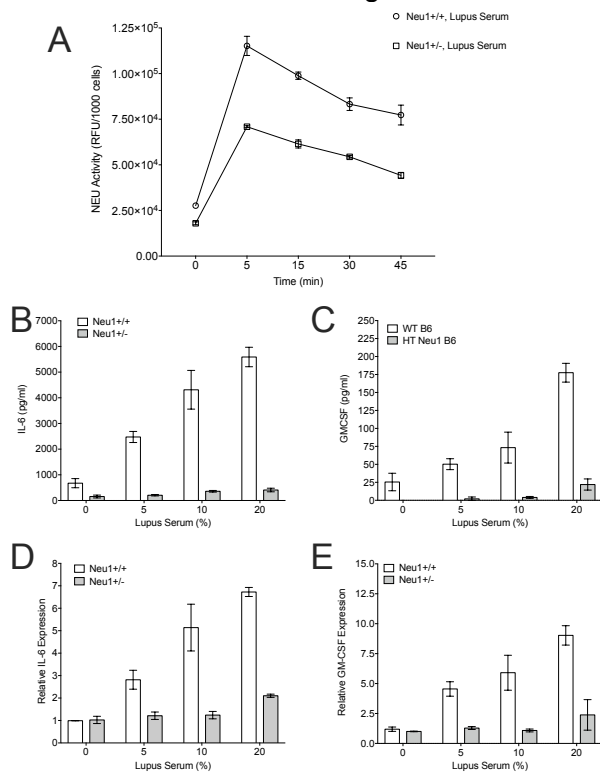


Fig. 4. MCs with a genetic knockout of one Neu1 allele (Neu1+/-) have reduced NEU activity, and secretion and expression of IL-6 and GM-CSF. Primary MCs were grown out of glomeruli isolated from 6 week-old C57BL/6 Neu1+/+ or Neu1+/- mice. A) MCs were cultured in the absence or presence of 20% lupus serum and NEU activity measured in live cells. MCs were cultured in the absence or presence of increasing percentage of lupus serum and secretion of IL-6 (B) and GM-CSF (C) into the media measured by ELISA and mRNA expression of IL-6 (D) and GM-CSF (E) in the cells measured by real-time RTPCR.

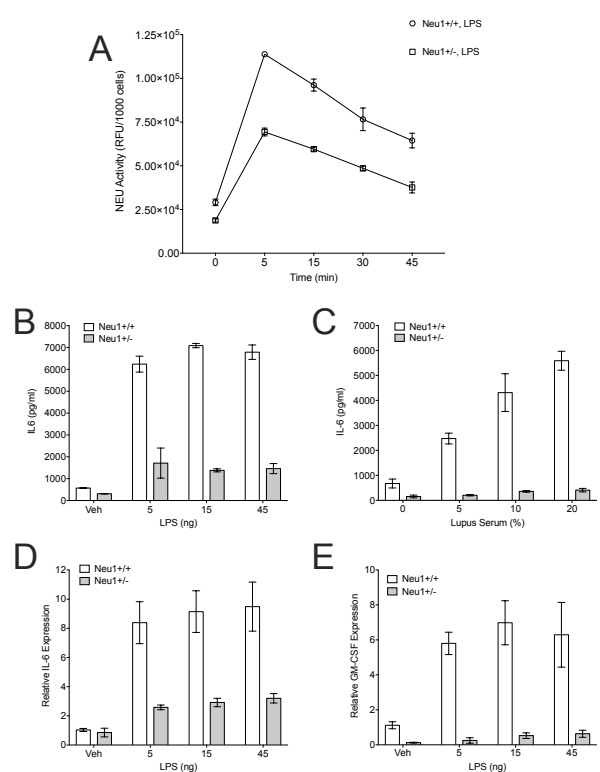


Fig. 5. MCs with a genetic knockout of one Neu1 allele (Neu1+/-) have reduced NEU activity, and secretion and expression of IL-6 and GM-CSF. Primary MCs were grown out of glomeruli isolated from 6 week-old C57BL/6 Neu1+/+ or Neu1+/- mice. A) MCs were cultured in the absence or presence of 5ng LPS and NEU activity measured in live cells. MCs were cultured in the absence or presence of increasing concentration of LPS and secretion of IL-6 (B) and GM-CSF (C) into the media measured by ELISA and mRNA expression of IL-6 (D) and GM-CSF (E) in the cells measured by real-time RTPCR.

Major Task 3: Exosomes were isolated from urine samples of lupus nephritis patients treated with mycophenolate mofetil prior to treatment and at 3 months and 12 months after treatment. Patients were divided into complete responders and non-responders to treatment. A total of 165 samples from 60 patients (30 complete responders, 30 non-responders) were included. All 60 patients had baseline (pre-treatment) and 3 months post-treatment samples, and 45 of the 60 patients had 12 month post-treatment samples. Glycosphingolipids HexCers and LacCers have been measured in all samples and the data is currently being analyzed. 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 10 “discovery” samples, and one previously identified biomarker of LN, are being confirmed using specific ELISAs. Proteins confirmed with the individual ELISAs will then be used to measure these proteins in the 165 samples. These studies are expected to be completed and a manuscript drafted and submitted within the no-cost extension period.

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

If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. “Training” activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. “Professional development” activities result in increased knowledge or skill in one’s area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

The PI attended the American Society of Nephrology Kidney Week and the American College of Rheumatology annual meetings in November, 2018 and presented research findings stemming from the DoD studies. The PI met with collaborators and other experts in the field to discuss approaches and ideas at both conferences.

How were the results disseminated to communities of interest?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

The PI presented at the American Society of Nephrology Kidney Week and the American College of Rheumatology annual meetings in November, 2018 on the preliminary results provided in the previous progress report and additional some of the preliminary results of the *in vivo* oseltamivir phosphate (OP) MRL/lpr mouse treatment study. Results from these studies were also presented locally to the PI’s collaborative lupus research group at MUSC and with the Rheumatology fellows as a means of educating the fellows on ongoing research questions and approaches being used to address these questions for translation to patient care.

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

If this is the final report, state “Nothing to Report.”

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

The project is in a one-year no-cost extension. Thus, we plan to complete the following before the final report:

Major Task 1: Successfully publish the manuscript describing the MRL/lpr treatment study recently submitted for review at the journal PLOS One, and successfully publish the manuscript describing the mechanisms by which NEU activity mediates IL-6 production by MRL/lpr lupus prone primary MCs through TLR4 and the p38/ERK MAPK pathway recently submitted for review at the Journal of Biological Chemistry.

Major Task 2: Complete the data analysis of the B6.SLE1/2/3 Neu1 mouse genetic study and the functional study comparing C57BL/6 Neu1+/+ and Neu1+/- primary MCs, finish drafting a manuscript describing these studies, and successfully publish the manuscript.

Major Task 3: Complete the urine exosome studies, data analyses, and manuscript preparation. The expectation is that these results will identify a panel of protein and GSL biomarkers in urine exosomes that will determine which LN patients are not likely to respond to or are not responding within 3 months of treatment with mycophenolate mofetil. A manuscript is expected to be submitted for review by the end of the no-cost extension period.

Major Task 4: We will continue moving forward with analyzing the patient renal biopsies by MALDI-FTICR, trouble-shooting the challenges of tissue integrity and continued issues with equipment maintenance and repairs. The expectation currently, is that we may obtain informative data from a limited number of the samples we have obtained, which we will include in the manuscript describing the studies in Major Task 3 as appropriate.

4. **IMPACT:** *Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

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

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

The combined results from the OP-treatment study in the MRL/lpr lupus strain and the B6.SLE1/2/3 Neu1+/- mouse genetic study suggest blocking the glycosphingolipid (GSL) catabolic pathway by inhibiting NEU activity or reducing levels of just NEU1 does not decrease GSLs (HexCers, LacCers, GM3s) or significantly impact disease. In fact, blocking all NEU activity in the MRL/lpr mice slightly elevates the already elevated levels of renal GSLs. Our *in vitro* results indicate NEU activity and NEU1 in particular is important in IL-6 production by mediating TLR4 signaling through p38/ERK MAPK pathways in mesangial cells. Thus, it is possible that NEU activity, and NEU1 in particular, are important in the production of IL-6 in lupus prone MCs during progression of lupus; however, broadly blocking/reducing NEU(1) activity *in vivo*, is not likely a viable therapeutic approach for LN.

Alternatively, blocking the GSL synthesis pathway may be more effective in slowing disease in lupus prone mice. This is important knowledge for the field of lupus, as this opens up additional and more focused avenues of research in targeting GSL metabolism as a potential treatment approach for lupus patients. The results from the urine exosome analyses will show that this is an informative and valid approach for identifying potential biomarkers of LN. Importantly, we will identify a panel of proteins and lipids that we anticipate can be used to identify patients that are likely to respond or not to mycophenolate mofetil treatment.

What was the impact on other disciplines?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

The results from the lupus mouse studies and urine exosome study are likely to have an important impact on other kidney diseases that exhibit altered GSL metabolism.

What was the impact on technology transfer?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to report

What was the impact on society beyond science and technology?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to report

- 5. CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

Changes in approach and reasons for change

Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

Nothing to report

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

Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Isolation and analysis of the urine exosomes in Major Task 3 has taken longer than anticipated. However, we are on track to finish up these analyses during the no-cost extension period.

The MALDI-FTICR analyses of the human biopsies from LN patients in Major Task 4 were delayed last year due to unforeseen issues with the MALDI equipment. We have since moved forward with these analyses only to discover that the integrity of most of the samples were compromised. Thus, the amount of data that can be obtained may be significantly limited. We are currently troubleshooting approaches to analyzing the tissue such that we can collect as much information about the lipids present in the biopsies as possible to obtain meaningful comparisons among renal biopsies from individuals with LN, diabetic nephropathy, polycystic kidney disease and no disease.

Changes that had a significant impact on expenditures

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to report

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

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

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: *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state “Nothing to Report.”*

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

Report only the major publication(s) resulting from the work under this award.

Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume; year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nowling TK, Rodgers J, Thiagarajan T, Wolf B, Bruner E, Sundararaj K, Molano I, Gilkeson G. Targeting glycosphingolipid metabolism as a potential therapeutic approach for treating disease in female MRL/lpr lupus mice. Submitted for review to PLOS One. (Federal support is acknowledged)

Sundararaj K, Rodgers J, Angel P, Wolf B, **Nowling TK**. Neuraminidase activity mediates IL-6 production through TLR4 and p38/ERK MAPK signaling in lupus serum-stimulated MRL/lpr mesangial cells. Submitted to Journal of Biological Chemistry. (Federal support is acknowledged)

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to report

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as*

noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.

T Nowling, J Rodgers, J Oates, M Janech, R Drake. 2018. Glycosphingolipid and Proteins in Urine Exosomes: Potential Biomarkers of Lupus Nephritis. American College of Rheumatology Annual Meeting, Chicago, IL.

T Nowling, K Sundararaj, J 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.

- **Website(s) or other Internet site(s)**

List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to report

- **Technologies or techniques**

Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to report

- **Inventions, patent applications, and/or licenses**

Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to report

- **Other Products**

Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

- *data or databases;*
- *physical collections;*
- *audio or video products;*
- *software;*

- *models;*
- *educational aids or curricula;*
- *instruments or equipment;*
- *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
- *clinical interventions;*
- *new business creation; and*
- *other.*

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate “no change”.

Example:

Name: Mary Smith
Project Role: Graduate Student
Researcher Identifier (e.g. ORCID ID): 1234567
Nearest person month worked: 5

Contribution to Project: Ms. Smith has performed work in the area of combined error-control and constrained coding.

Funding Support: The Ford Foundation (Complete only if the funding support is provided from other than this 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 the MALDI-FTICR analyses on the renal biopsies 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 and the *in vitro* mesangial cell analyses.

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?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Tamara K. Nowling, PhD: No changes.

James C. Oates, MD: In the past year, Dr. Oates provided effort on one new study:

R01 AI138511-1A1

Vasu (PI)

11/06/18-10/31/2023

Role of microbiota-TLR7/8 Interaction in systemic lupus erythematosus

Goal(s): determine the role of TLR7/8 overexpression and the interactions of these receptors with gut microbiota in causing intestinal inflammation, pre-Hp2 expression, and autoantibody production that lead to systemic autoimmunity in lupus using human samples and mouse models. Role: Co-Investigator

Bethany Wolf, PhD: In the past year, Dr. Wolf provided effort on one new study:

R03 TR003038 (PI: Nihalani) 8/1/2019-7/31/2021

NIH/NCATS \$74,750

Clinical Assessment of a Novel Non-Invasive Assay for Diagnosing Recurrent Focal and Segmental Glomerulosclerosis

The primary goal of this R03 proposal is to develop a novel cell-based assay that will serve as a non-invasive diagnostic clinical tool to detect a rare form of glomerular disease commonly known as recurrent focal and segmental glomerulosclerosis (rFSGS).

Role: Co-Investigator

Richard Drake, PhD: No changes

What other organizations were involved as partners?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:

Organization Name:

Location of Organization: (if foreign location list country)

Partner’s contribution to the project (identify one or more)

- *Financial support;*
- *In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);*
- *Facilities (e.g., project staff use the partner’s facilities for project activities);*
- *Collaboration (e.g., partner’s staff work with project staff on the project);*
- *Personnel exchanges (e.g., project staff and/or partner’s staff use each other’s facilities, work at each other’s site); and*
- *Other.*

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to <https://ers.amedd.army.mil> for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on <https://www.usamraa.army.mil>) should be updated and submitted with attachments.*

9. **APPENDICES:** *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*
- **Nowling TK**, Rodgers J, Thiyagarajan T, Wolf B, Bruner E, Sundararaj K, Molano I, Gilkeson G. Targeting glycosphingolipid metabolism as a potential therapeutic approach for treating disease in female MRL/lpr lupus mice. (Manuscript submitted for review to PLOS One)
 - Sundararaj K, Rodgers J, Angel P, Wolf B, **Nowling TK**. Neuraminidase activity mediates IL-6 production through TLR4 and p38/ERK MAPK signaling in lupus serum-stimulated MRL/lpr mesangial cells. (Manuscript submitted to Journal of Biological Chemistry)
 - **T Nowling**, J Rodgers, J Oates, M Janech, R Drake. 2018. Glycosphingolipid and Proteins in Urine Exosomes: Potential Biomarkers of Lupus Nephritis. American College of Rheumatology Annual Meeting, Chicago, IL. (Abstract, poster presentation)
 - **T Nowling**, K Sundararaj, J 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. (Abstract, poster presentation)

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

Short title: Targeting glycosphingolipid metabolism in lupus mice

Tamara K. Nowling^{1*}, Jessalyn Rodgers¹, Thirumagal Thiyagarajan¹, Bethany Wolf², Evelyn Bruner³, Kamala Sundararaj¹, Ivan Molano¹, Gary Gilkeson¹

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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 slightly increased, rather than decreased, renal 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, 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

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). Ceramides (Cers), hexosylceramides (HexCers), lactosylceramides (LacCers), sphingomyelins (SMs), Sphingosine-1 Phosphate (S1P), Sphingosine (So), Dihydrosphingosine-1 Phosphate (DHS1P), and Dihydrosphingosine (DHSo) were quantified 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). 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 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.

Serum IgG ELISA

Total IgG levels in mouse sera were measured by ELISA as described previously (23). 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 (23) 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.

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.

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). There were also no differences in the scores of the individual pathological features between the water- and OP-treated lpr groups (data not shown).

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 hyperactivation *ex vivo* (23). 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 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/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 (24), 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. Figure 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 renal C24 Cer levels only, but 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). The increased levels of HexCers were due to 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. Urine Cers, HexCers, and LacCers were also measured in the urine of the first set of MRL/lpr water- and OP-treated mice in pre-treatment and endpoint samples. Results showed all three lipids

increased from pre-treatment to endpoint with slightly (but not significantly) higher levels of in the OP-treated mice compared to the water-treated mice HexCers at the endpoint (data not shown), similar to what was observed for HexCers in the kidney (Fig. 4B).

Since we did not observe significant effects of OP on renal GSL levels, we examined the effect of OP treatment on renal NEU activity with the expectation that the OP-treated mice would have reduced renal NEU activity. Both the water-treated and OP-treated lpr mice had a significant increase in NEU activity over time (Fig. 4D, $p < 0.001$). However, NEU activity levels were not significantly different between the water- and OP-treated lpr mice. MpJ mice did not show a significant increase in NEU over time and had significantly different NEU levels relative to both water- and OP-treated lpr mice (adjusted p-values for water vs. MpJ at 30, 45, 60, 75, and 90 minutes respectively: 0.720; 0.059; < 0.001 ; < 0.001 ; < 0.001) as expected based on previous observations(13). Together, the results of this OP treatment study suggest 10 mg/kg OP daily for four weeks does not significantly block renal NEU activity or significantly impact GSL levels.

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 (25, 26). 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. 5A). 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. 5A, 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. 5A). 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. 5B) and with all GM3 chain lengths at $p < 0.01$ except C24:1, which was at $p < 0.05$ (Fig. 5C). When pairwise comparisons among doses were evaluated, decreases for each LacCer chain length did not reach significance (Fig. 5B) and only C16 GM3 at 10mg and 20mg doses compared to 0 mg reached significance (Fig. 5C, p-values indicated on graph). There were no significant differences in Cers C16, C22, C24, C24:1, or total Cer with any dose (Fig. 5D).

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 (data not shown). Renal pathology score (Fig. 5E) was not significantly associated with dose of Genz-667161 (treating dose as either continuous- $p=0.112$, or categorical- $p=0.219$). However, spleen to body weight ratio was negatively correlated (Fig. 5F, $\rho=-0.535$, $p=0.040$) and serum IgG levels were marginally correlated (Fig. 5G, $\rho=-0.493$, $p=0.062$) 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 (27-30) and in lupus mice (23, 31). 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 (23). 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 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 found to be elevated for many more 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.

Based on the observed increase in NEU activity in the MRL/lpr nephritic kidney (13), we hypothesized that blocking NEU activity may normalize GSLs levels and improve disease. 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 10-50-fold higher than Neu3 in human and mouse while Neu2 and Neu4 are expressed at low to undetectable levels in the kidney (14, 32-34). 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 mouse kidney upregulate Neu1 expression and NEU activity while blocking NEU activity significantly decreases cytokine release upon *ex vivo* stimulation (14) (unpublished data, Sundararaj and Nowling). Together, these results suggested elevation of renal HexCers and LacCers may be due to increased break down of gangliosides by NEUs. However, our results here blocking NEU activity and the catabolic pathway using the NEU inhibitor OP resulted in a trend towards increased GSLs. Although it was not significant, the trend

towards increased HexCers, LacCers, and GM3s indicate blocking the catabolic pathway results in an accumulation of GlcCers, LacCers, and GM3. This is likely due to their continued *de novo* synthesis from Cer. Based on these results, the significant increase in renal NEU activity (13, 14) may be a response to elevated GSL levels and attempt to reduce GM3 accumulation. The observed slight increases in renal GM3s, HexCers, and LacCers when NEU activity is inhibited in OP-treated mice further supports this hypothesis.

The lack of significant effects on either the levels of GSLs or disease measures in the OP treatment study may be due to several factors/limitations of the study. First, OP is a prodrug for the active metabolite oseltamivir carboxylate (OC), a structural homolog of sialic acid developed as a competitive inhibitor of viral NEU and is not as effective against mammalian NEUs. However, several studies, including our own, demonstrated it blocks 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) (14, 20, 35-39). 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 (Karhadkar, 2017); however, higher doses may be required to have significant effects in the kidney. Second, 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. Hence, the lack of significant changes in GSLs and disease effects in the OP-treated group compared to the water-treated group may be a result of insufficient dosing. Specific mammalian NEU inhibitors are needed to

accurately determine how/if blocking all NEU activity or the activity of a specific NEU will impact 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. In addition, 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. 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 by the breakdown of accumulated SMs, So and 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 synthesis pathway may be more effective than blocking the catabolic pathway in normalizing GSL metabolism in nephritic lupus mice and may be translationally relevant to human patients with lupus nephritis who have elevated urine GSLs levels (13). A specific mammalian NEU inhibitor would likely provide a clearer answer to whether blocking GSL catabolism will normalize GSL metabolism and improve disease in LN. Alternatively, blocking both pathways may be necessary to effectively normalize GSL metabolism and improve disease expression/progression in lupus nephritis.

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Figures

Figure 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.

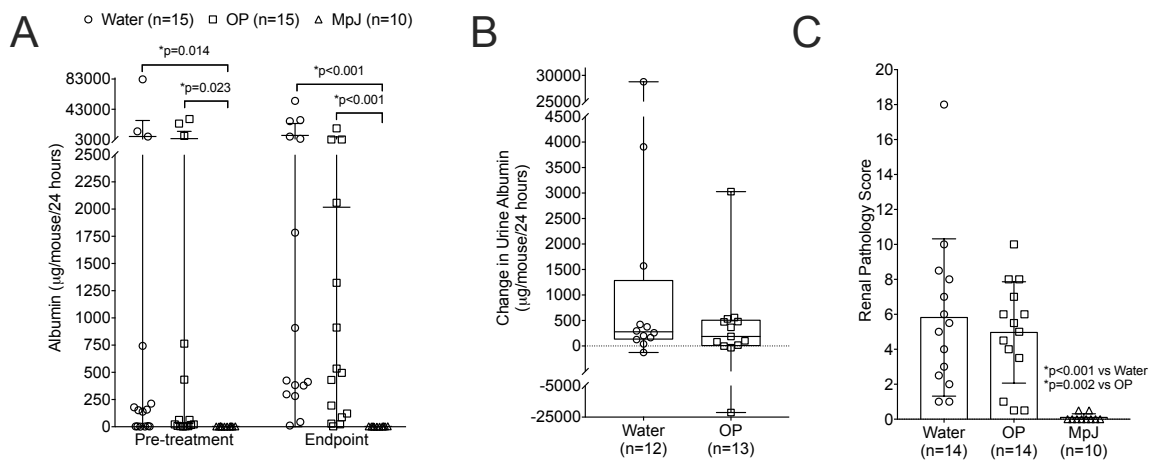


Figure 2. Systemic disease measures are not significantly impacted by OP treatment in MRL/lpr mice. Splenomegaly in the same mice as in Fig. 1 was determined by absolute spleen weight (A) and as spleen to body weight ratio (B). C) Percentages of activated/memory T cells ($CD3^+ CD44^+ CD62L^-$) were 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.

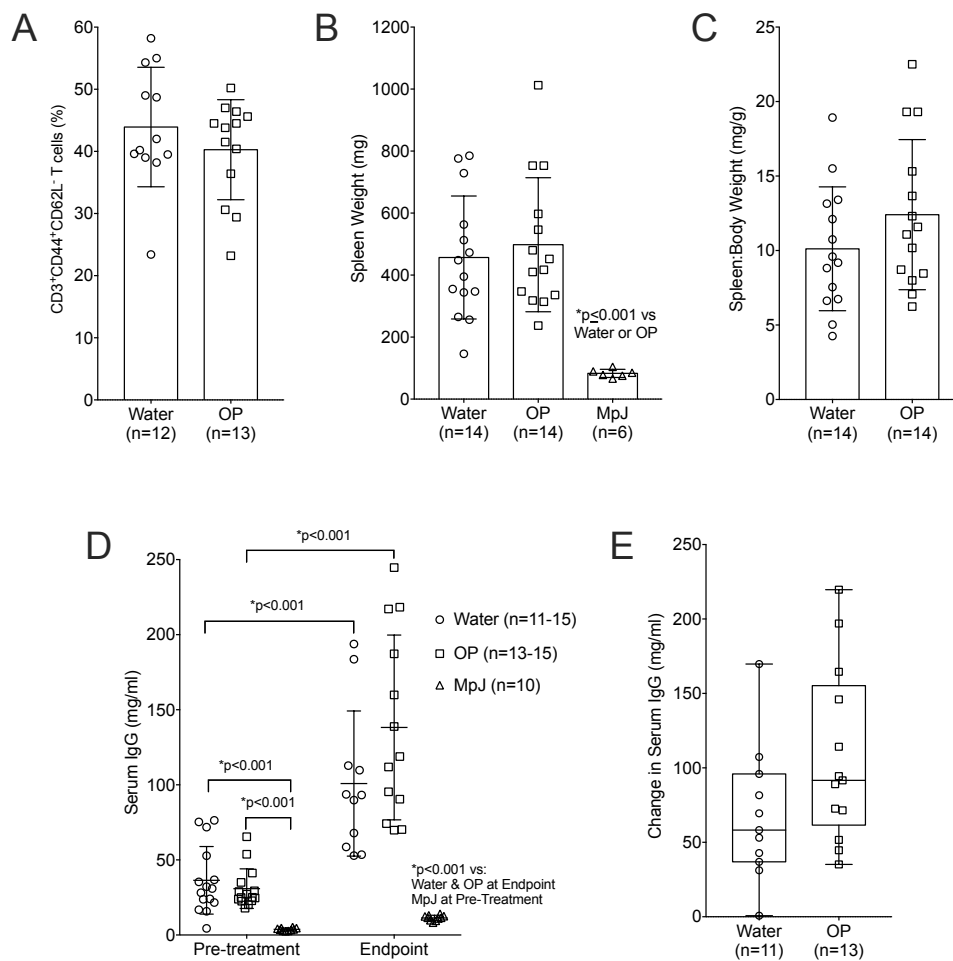


Figure 3. Renal GM3s, SMs, and Sphingolipids are elevated in nephritic MRL/lpr compared to non-nephritic age-matched MRL/MpJ mice and not significantly impacted by treatment with OP. Renal lipids were quantified in renal cortex homogenates from the same mice as in Fig. 1 at the endpoint of the study. 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.

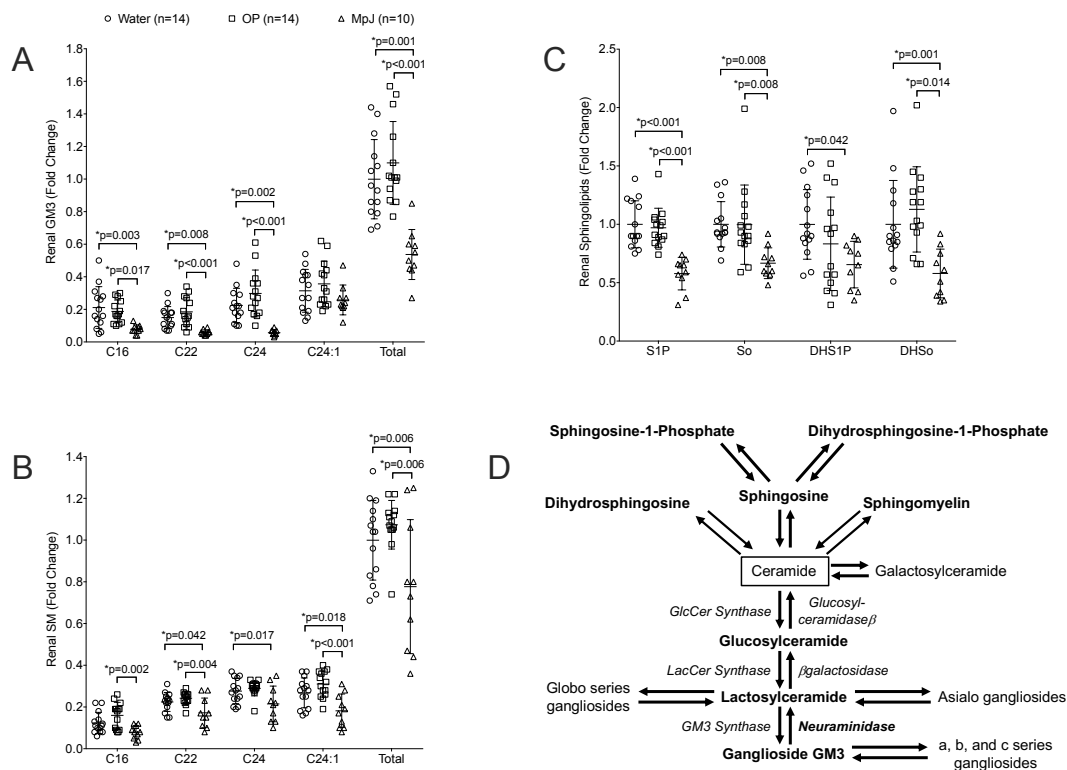


Figure 4. Renal Total Cers, HexCers, and LacCers are slightly increased and NEU activity is unchanged in the OP-treated compared to water-treated MRL/lpr mice.

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. 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.

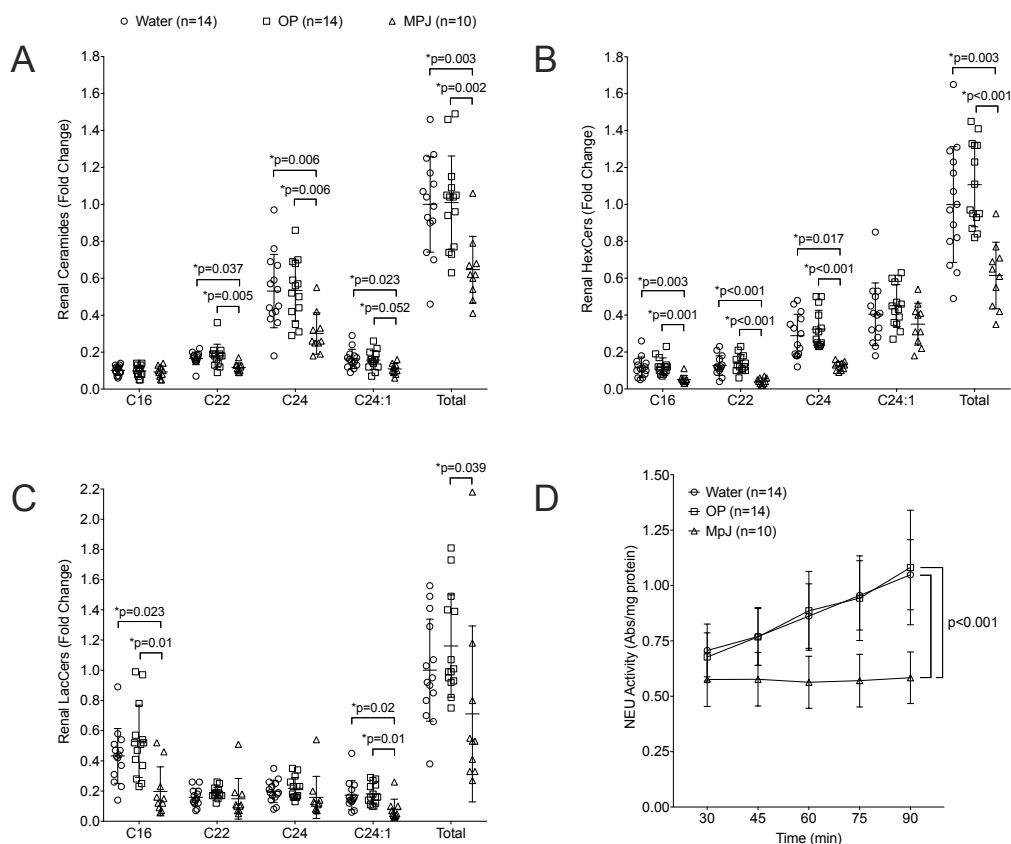
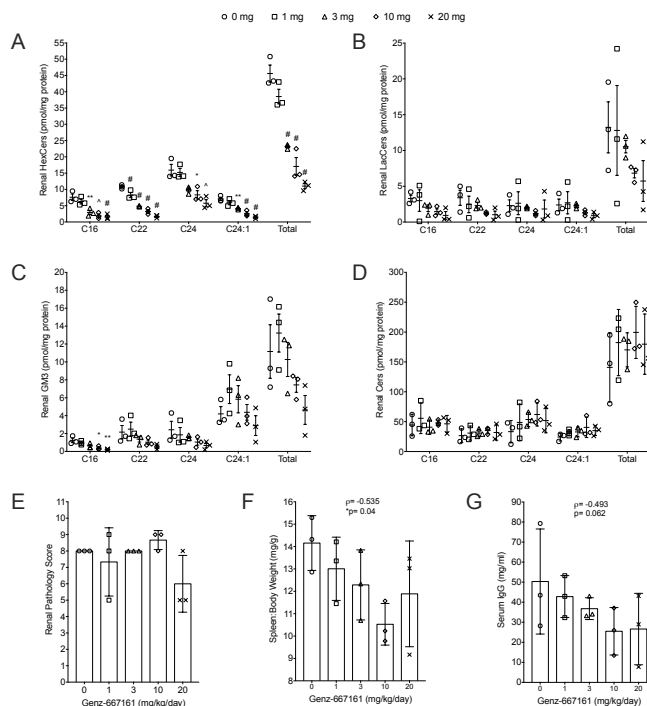


Figure 5. Renal HexCers, LacCers, GM3s, splenomegaly, and serum IgG are decreased with increasing dose of a GlcCer synthase inhibitor in MRL/lpr mice.

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: * $p \leq 0.05$, ** $p \leq 0.01$, $^{\wedge}p \leq 0.005$, $^{\#}p \leq 0.001$. 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.



TITLE

Neuraminidase activity mediates IL-6 production through TLR4 and p38/ERK MAPK signaling in lupus serum-stimulated MRL/lpr mesangial cells

Running Title: Mechanisms of IL-6 expression in lupus MCs

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ABSTRACT

Mesangial cells (MCs) are considered the immune cell of the kidney, receptor dependent and independent uptake of molecules such as immunoglobulins, and secreting a number of cytokines including IL-6, MCP-1, CSF-1, and TNF α . IL-6 serves as an autocrine factor for MCs to stimulate proliferation, and is associated with disease in patients and mouse strains with various kidney diseases. With respect to lupus nephritis, IL-6 was shown to promote tissue damage. glycosphingolipid (GSL) metabolism in the kidneys of lupus mice and human patients with nephritis is altered characterized by elevated several GSLs and activity of the GSL catabolic enzyme neuraminidase (NEU). To delineate the role of NEU at the cellular level in lupus nephritis, we previously demonstrated increased IL-6 secretion from primary MCs from lupus prone MRL/lpr mice in response to the immune complex mimic heat aggregated IgG or lupus serum that was mediated by NEU activity. Here we further those studies to demonstrate that lupus serum- or LPS-stimulated lupus prone primary MCs from MRL/lpr mice results in increased NEU activity, cytokine secretion of IL-6, GM-CSF, and MIP1 α that is reduced by an inhibitor of NEU activity. Moreover, the expression of all three cytokines at the message level also increases in response to lupus serum and LPS, but only IL-6 expression is reduced when NEU activity is inhibited. We also show that TLR4 and p38/ERK MAPK signaling play a role in the NEU-mediated IL-6 secretion and blocking NEU activity increases the levels of GSLs and sialylated glycoproteins. Together, our results suggest NEU plays an important role in MC response to a circulating factor that stimulates IL-6 expression and secretion requiring TLR4-p38/ERK MAPK signaling, likely through desialylation of one or more glycoproteins in this pathway.

INTRODUCTION

Systemic lupus erythematosus (SLE) is an inflammatory, chronic autoimmune disorder involving multiple organs. Defects in the clearance of apoptotic cells lead to intracellular autoantigens 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 cytokine IL-6 is upregulated in the kidney of lupus mice and patients with lupus and was shown to be important for onset of nephritis in lupus mice (6-11). The 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 *in vitro* (12).

Neuraminidases (NEUs) (or sialidases) remove sialic acids from glycolipids and glycoproteins. There are four mammalian NEUs. NEU1 is typically located in the lysosome and was shown to be 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 Neu1 being expressed approximately 40-fold higher than Neu3 at the message level (19) and we readily detect both NEU1 and NEU3 in glomeruli in tissue sections of MRL/lpr lupus mice (12). While NEU1 was largely observed to desialylate glycoproteins (25), NEU3 showed preference for gangliosides (26-28) and their activity at the plasma membrane impacts receptor activation and signaling in a variety of cell types (28-34).

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 of MCs, we determined that 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 these cytokines occurs in part through TLR4-MAPK p38 or ERK signaling pathways in response to lupus serum and TLR4 ligand LPS that is likely due to removal of sialic acid residues from cell surface signaling proteins in this pathway by NEU.

MATERIALS AND METHODS

Reagents

Antibodies used include anti-Fc γ R3 and anti-TNFR1/TNFRSF1A (R&D Systems, Minneapolis, MN); anti-Fc γ R2/3, anti-Fc γ R4, anti-TLR2, and anti-TLR4/MD2 (BioLegend, San Diego, CA); anti-TNFR2 (BioXCell, West Lebanon, NH); anti-Annexin A2 (Abcam, Cambridge, MA); and isotype controls hamster IgG and mouse IgG (BioLegend, San Diego, CA), and rat IgG (Rockland Immunochemicals, Limerick, PA). 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). Cell stimulants include: Ultra-pure Lipopolysaccharide (LPS) (Invivogen, San Diego, CA); Lupus serum was obtained from 16-18 week-old MRL/lpr mice and pooled. Each pooled serum collection was tested for endotoxin using Pierce Chromogenic Endotoxin Quantification Kit (Thermo Scientific, Rockford, IL). All MRL/lpr serum (lupus serum) used for experiments had undetectable levels of endotoxin.

Generation and culturing of primary mesangial cell lines

Kidney glomeruli were isolated from 6-week-old pre-nephritic female MRL/lpr mice for generation of two independent primary mesangial cell lines (MCs) as described previously (12). Each primary MC line was characterized by flow cytometry at passage 5, by IHC at passage 6, and by RT-PCR at passages 5–9 as performed previously (35) and were 93% positive for α SMA by flow cytometry. All cell lines tested negative for mycoplasma (Applied StemCell, Inc, Milpitas, CA). 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 μ g/ml insulin, 5 μ g/ml transferrin, 5 ng/ml sodium selenite)(GenDEPOT, Houston, TX; Sigma-Aldrich, St. Louis, MO) and cell

passages 6-9 were utilized for all experiments. Cells were pretreated with 500 μ M OP for 16 hrs, 40 μ g/ml antibodies for 2 hrs, and 10 mM MAPK/PI3K inhibitors for 2 hrs prior to stimulation with 5-20% lupus serum or 5-45 ng/ml LPS for 0.5-24 hrs as indicated in the figure legends. Results from each line showed similar trends with data presented from one line.

Neuraminidase activity assay

Primary MCs (20,000 cells per well) were grown in a CoStar 3603 black 96-well clear-bottom plate, pre-treated with 500 μ M OP and stimulated with 10% lupus serum/ 5ng/ml LPS for 30 min, 1 h, and 3 h . After incubation, media was collected, and cells were washed with 37°C warmed sterile filtered Tris-buffered saline (TBS) pH 7.4. Neuraminidase activity was measured using a SpectraMax i3c fluorometer with SoftMax Pro 6 software (Molecular Devices, San Jose, CA). Cells were incubated with 15 μ M 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 at baseline prior to substrate addition and in the presence of substrate as indicated on the graph. Since total protein content was consistent between untreated and treated cells, the results are reported as RFU per 20,000 cells.

FlexMap 3D and ELISA analyses

To determine cytokines and chemokines in addition to IL-6 potentially mediated by NEU activity, primary 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. ELISAs were used 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, MIP1 α and housekeeping gene b-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: mL6 Forward 5'-TAGTCCTTCCTACCCCAATTTCC-3' and Reverse 5'-TTGGTCCTTAGCCACTCCTTC-3'; GM-CSF Forward 5'-GGCCTTGGAAGCATGTAGAGG-3' and Reverse 5'-GGAGAACTCGTTAGAGACGACTT-3'; mMIP1 α Forward 5'-ACTGCCTGCTGCTTCTCCTACA-3' and Reverse 5'-AGGAAAATGACACCTGGCTGG-3'; m β 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.

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/ThermoScientific, 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 anti-mouse total and anti-mouse phosphorylated MAPK primary antibodies (Cell Signaling Technology, Danvers, MA), respectively. Detection was further enhanced with an anti-rabbit biotin antibody (Thermo Scientific, Rockford, IL) and 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 Image Version 1.63. The results are presented as the ratios of phosphorylated MAPK to total MAPK.

Lipidomics and N-glycan analyses

Ceramides (Cers), hexosylceramides (HexCers), lactosylceramides (LacCers), sphingomyelins (SMs), Sphingosine-1 Phosphate (S1P), Sphingosine (So), Dihydrosphingosine-1 Phosphate (DHS1P), and

Dihydrosphingosine (DHSO), and Ganglioside GM3s were quantified by the Virginia Commonwealth University (VCU) Lipidomics/Metabolomics Core Facility as described previously (36) in cell homogenates prepared as described previously (12). Lipid levels were measured for all chain lengths and total for all chain lengths for each lipid normalized to cell number is presented as pg/10⁶ cells. N-glycans were measured in intact cells grown on glass slides as follows. Cells were plated 24 hrs prior to addition of OP or vehicle (water). Cells were incubated with OP for 16 hrs and media replaced containing no lupus serum, 10% lupus serum, or 10% heat-treated lupus serum for 3 hrs. Heat-treated lupus serum was incubated at 56C for 30 min. Heat-treated lupus serum did not stimulate IL-6 secretion (data not shown).

Statistical analyses

Statistical analyses were performed using GraphPad PRISM 7 software (GraphPad Software, La Jolla, CA).

RESULTS

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

To better define the effects of lupus serum in stimulating cytokine expression by lupus prone MCs, Multiplex FlexMap3D bead array analysis was used to measure a panel of twenty cytokines. MRL/lpr MCs were cultured in the presence of serum taken from healthy non-autoimmune prone C57BL/6 (B6) mice or MRL/lpr nephritic mice. Eleven cytokines were increased by at least 2-fold after addition of lupus serum compared to B6 serum. In addition to IL-6 secretion, which we demonstrated previously is significantly increased by lupus serum-stimulated MCs, GM-CSF and MIP1 α were highly elevated in the media of lupus MCs 6 hrs after addition of lupus serum (data not shown). Individual ELISAs confirmed, in addition to IL-6 (Fig. 1A) as we previously demonstrated (12), MC secretion of GM-CSF (Fig. 1B) and MIP1 α (Fig. 1C) was significantly increased dose-dependently by lupus serum 6 hrs after stimulation. Next, we analyzed the temporal release of these cytokines with respect to lupus serum stimulation. Media from lupus MCs stimulated with lupus serum for 1, 3 and 6 hrs shows release of all three cytokines is significantly increased within 3 hrs of stimulation (Fig. 1D-F). IL-6 (Fig. 1G) and GM-CSF (Fig. 1H) message levels are also significantly increased following lupus serum stimulation. GM-CSF mRNA levels were increased within 30 min of stimulation (Fig. 1H). Mip1 α mRNA levels were

undetectable in unstimulated cells and remained at the threshold of minimal detection after stimulation (Fig. 1I) depending on the experiment. Thus, we were unable to conclude with any degree of certainty if MIP1 α expression was increased following lupus serum stimulation.

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

Previously, we showed lupus serum stimulates IL-6 secretion by MRL/lpr primary MCs and blocking NEU activity by pretreating cells with the oseltamivir phosphate (OP), a NEU inhibitor, prior to addition of stimulation significantly reduced IL-6 secretion. Results from the FlexMap3D experiment indicate that GM-CSF and MIP1 α may also be mediated by NEU activity. MCs were pretreated with OP or vehicle (water) and stimulated with lupus serum and cytokine levels measured in the media by ELISA. This experiment confirmed all three cytokines were significantly decreased when MCs were pretreated with OP prior to lupus serum stimulation (Fig. 2A-C). Interestingly, OP treatment blocked the increase in Il-6 mRNA levels (Fig. 2D), but not the increase in Gm-csf mRNA levels (Fig. 2E) by lupus serum. In fact, pretreating with OP further increased Gm-csf mRNA levels following lupus serum stimulation. To confirm that OP is blocking NEU activity, a live-cell NEU activity assay was performed on lupus serum-stimulated MCs in the absence and presence of OP (Fig. 2F). These results suggest NEU activity may mediate IL-6 secretion by blocking its expression at the message level. The mechanism by which inhibition of NEU activity blocks GM-CSF secretion while potentially further increasing message levels is yet to be determined.

TLR4 signaling plays a role in NEU-mediated IL-6 release in MRL/lpr MCs

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 (37-41). Therefore, the role of these proteins in lupus serum-stimulated primary MCs was investigated. MCs were incubated with neutralizing antibodies for TLR2, TLR4/MD2, Fc γ R2/3, Fc γ R3, Fc γ R4, AnxA2, and TNFRs to block their interaction with putative ligands prior to stimulation with lupus serum. TLR4/MD2 neutralizing antibody significantly inhibited

lupus serum-stimulated IL-6 secretion, while antibodies to TLR2, Fc γ receptors, AnxA2, TNFRs, and TLR4 only (and not MD2) failed to reduce IL-6 release (Fig. 3A and data not shown). Secretion of GM-CSF was slightly reduced and MIP1 α was not significantly impacted by anti-TLR4/MD2 antibodies but were otherwise not impacted by any of the antibodies tested (Fig. 3B-C and data not shown). Isotype control IgGs for each of the antibodies tested did not reduce release of any IL-6, GM-CSF, or MIP1 α (data not shown).

To determine if NEU activity mediates cytokine release through TLR4, the TLR4 ligand LPS was used to stimulate primary MRL/lpr MCs. As with lupus serum, 5 ng/ml LPS stimulated release of IL-6 (Fig. 4A), GM-CSF (Fig. 4B), and MIP1 α (Fig. 4C). Increasing the concentration of LPS did not further increase the levels observed at 5 ng/ml indicating that release was maximal at this concentration. Thus, 5 ng/ml LPS was used to stimulate cells for all subsequent experiments. The release and mRNA expression of these cytokines by LPS stimulation followed a similar temporal trend as that observed following lupus serum stimulation. Significant increases in all three cytokines was observed in the media within 3 hrs of LPS addition (Fig. 4D-F). IL-6 (Fig. 4G) and GM-CSF (Fig. 4H) mRNA levels increased within 1 hr and 30 min, respectively, while MIP1 α mRNA levels were essentially undetectable even after stimulation (data not shown). Importantly, inhibiting NEU activity with the inhibitor OP, significantly reduced LPS-stimulated release of IL-6, GM-CSF, and MIP1 α (Fig. 5A-C) and mRNA expression of IL-6 and GM-CSF (Fig. 5D and E), as observed in lupus serum-stimulated MCs. To confirm that OP is blocking NEU activity, a live-cell NEU activity assay was performed on lupus serum-stimulated MCs in the absence and presence of OP (Fig. 5F). These results demonstrate TLR4 activation of IL-6, GM-CSF, and MIP1 α release by MCs and mRNA expression of IL-6 and GM-CSF are mediated by NEU activity, suggesting NEU mediates TLR4 signaling in upregulating expression, as well as release, of IL-6 and GM-CSF.

To confirm that TLR4 is required for the LPS-stimulated release of IL-6, GM-CSF and MIP1 α , neutralizing antibodies to TLR4/MD2, as well as the other cell surface receptors tested in lupus serum-stimulated MCs in Fig. 3, were incubated with MCs prior to addition of LPS. TLR4/MD2 neutralizing antibody significantly blocked LPS-stimulated IL-6 (Fig. 6A) and GM-CSF (Fig. 6B) secretion, whereas TLR2 and Fc γ R_s had no effect. None of the neutralizing antibodies, including TLR4/MD2, had an effect on MIP1 α secretion. Together, these results indicate NEU-mediated release of IL-6 and GM-CSF from lupus prone MCs occurs in part through TLR4 activation, while

NEU-mediated secretion of MIP1 α occurs through a pathway that does not appear to require TLR2, TLR4, Fc γ Rs, TNFRs, or AnxA2.

NEU activity mediates TLR4-MAPK signaling in the release of IL-6 in stimulated MCs

TLR4 receptor can signal through MAPK and PI3K pathways. Thus, to determine if the MAPK (ERK, JNK, p38) or PI3K pathways play a role in NEU-mediated lupus serum-stimulated release of IL-6 in MCs, inhibitors for each pathway were first employed. These included U0126, SB203580, and SP600125, inhibitors of ERK, p38, and JNK respectively, and an inhibitor of PI3K, wortmannin. ERK and p38 MAPK inhibitors significantly reduced lupus serum-stimulated IL-6 secretion approximately 50% and 40%, respectively (Fig. 7A) and LPS-stimulated IL-6 secretion approximately 70% and 50%, respectively (Fig. 7B). Although blocking TLR4 did not have an effect on GM-CSF release in lupus serum-stimulated MCs, it did significantly reduce GM-CSF release from LPS-stimulated MCs. Inhibiting ERK signaling significantly reduced GM-CSF release when stimulated with either lupus serum (Fig. 7A) or LPS (Fig. 7B). None of the inhibitors reduced MIP1 α release from MCs when stimulated with lupus serum (Fig. 7A) and LPS (Fig. 7B) and actually further increased MIP1 α secretion depending on the inhibitor.

To determine if NEU activity plays a role in ERK and p38 MAPK signaling in lupus serum-stimulated MCs, MRL/lpr primary MCs were incubated with OP followed by stimulation with lupus serum for 15, 30, and 45 min. Within 15 min of lupus serum stimulation, activation of both ERK (Fig. 7C) and p38 (Fig. 7D) was increased as determined by the amount of phosphorylated ERK and p38. This increase was blocked by the NEU inhibitor OP at 45 min, indicating NEU activity mediates ERK and p38 MAPK signaling in response to lupus serum. This experiment was repeated in LPS-stimulated MCs to examine the role of NEU activity in ERK and p38 MAPK signaling specifically through TLR4 activation. As in the lupus serum-stimulated MCs, ERK and p38 phosphorylation (activation) was observed within 15 min of LPS stimulation, and inhibiting NEU activity with OP reduced ERK and p38 activation at 30 min following activation (Fig. 7E-F). Although there was some variation in the extent of ERK and p38 activation from experiment to experiment, depending on the lupus serum lot used for stimulation, the observed reduction in levels in the presence of OP was consistent at 45 min for lupus serum-

stimulated and at 30 min for LPS-stimulated MCs. Together, these results suggest NEU mediates TLR4-ERK/p38 MAPK signaling in lupus serum stimulated lupus MCs.

NEU catabolism of N-glycans likely plays a role in the TLR4-MAPK IL-6 release by lupus serum-stimulated MCs.

Sialic acid containing glycolipids, such as gangliosides, and glycoproteins serve as substrates of NEUs. We previously reported that elevating glycosphingolipids (GSLs) glucosylceramide (GlcCer) and lactosylceramide (LacCer) stimulate increases in the expression of several cytokines, including IL-6, in the immortalized mouse MES13 MC line (12). To determine if NEU mediates cytokine release through regulation of GSL levels, GlcCer, LacCer, and GM3 (the simplest ganglioside generated from LacCer) were measured in lupus serum-stimulated MCs in the absence or presence of OP. HexCers and GM3s were significantly elevated upon stimulation and, unexpectedly, further elevated in the presence of OP (Fig. 8A). Also, although they were not elevated upon stimulation, LacCer and ceramide levels were significantly elevated in the presence of OP. Due to the elevated levels of ceramide in the presence of OP, levels of lipids that feed into ceramide and the GSL synthesis pathway were also measured. Dihydrosphingosine (DHSO) was increased upon stimulation and further significantly elevated in OP-treated cells (Fig. 8B). Sphingosine (So) was also significantly elevated in stimulated cells treated with OP (Fig. 8B) and suggests blocking GSL catabolism causes an accumulation of GSLs due to continued synthesis through ceramide and lipids feeding into ceramide. Increasing GlcCer and LacCer levels stimulated IL-6 expression (in the absence of additional stimulants) (12), and blocking NEU activity increased GlcCer and LacCer levels yet reduced IL-6 expression. Thus, NEU activity does not appear to mediate IL-6 expression by altering GlcCer and LacCer levels. We also analyzed effects of lupus serum stimulation and inhibition of NEU activity on the N-linked glycoproteins, which are. The overall levels of sialylated N-glycans are not significantly altered when MCs are stimulated with lupus serum and, as expected, are significantly elevated when NEU activity is blocked by OP (Fig. 8C). These results suggest that inhibiting the removal of sialic acids from N-glycans may prevent the interaction of ligands in the lupus serum with receptors on the surface of MCs.

DISCUSSION

We previously demonstrated that neuraminidase (NEU) activity could mediate the release of IL-6, but not MCP-1, from lupus prone MRL/lpr mesangial cells (MCs) following stimulation with an immune complex mimic (heat-aggregated IgG) (12). Here we extended those studies to examine the expression and release of IL-6 and other cytokines in these cells following treatment with serum collected from nephritic MRL/lpr mice, a disease-relevant stimulant. Together these studies indicate release of IL-6 by lupus serum-stimulated MCs is likely directly mediated by NEU activity through TLR4-MAPK p38 or ERK signaling since both IL-6 message levels and release were significantly decreased when NEU activity was inhibited. Lupus serum-stimulated secretion of MIP1 α and GM-CSF were also significantly decreased when NEU activity was inhibited. However, their expression at the message level was not decreased, suggesting NEU activity indirectly mediates their secretion. Both anti-TLR4 antibodies and MAPK p38 or ERK inhibitors prevented cytokine release of IL-6 following lupus serum stimulation. Both p38 and ERK were phosphorylated upon lupus serum-stimulation, which was decreased when NEU activity was inhibited. These results were confirmed when using LPS stimulation to isolate the TLR4 signaling pathway, supporting a role for NEU activity in mediating IL-6 upregulation and release through TLR4-MAPK p38/ERK signaling pathways.

Following LPS binding, optimal TLR4 signaling requires MD2 and CD14 forming a TLR complex. Both TLR4 and MD2 contain multiple N-glycans that are sialylated and required for function (42-44). SAs mask binding epitopes of receptors and SA removal enhances binding and subsequent signaling through TLR4 on leukocytes (44), DCs (45), PBMCs (30), and HEK293T cells (44,46). Desialylation of both TLR4 and MD2 by NEU1 enhances TLR4 dimerization and signal transduction in response to LPS in embryonic HEK293T cells (44). TLR4 is readily detected on MCs from MRL/lpr, but not from healthy non-autoimmune BALB/c mice (38). TLR4-deficient C57BL/6 lpr/lpr lupus mice have attenuated renal disease (47). NEU1 was shown to be a key regulator of ligand-induced TLR(4) activation in the function of macrophages, with TLR-ligand interaction rapidly activating NEU1 (31,32). TLR4 diffuses into lipid rafts upon LPS stimulation (48) to propagate signaling of gene expression.

Alternatively or additionally, both glycoproteins and glycolipids may play a role in modulating TLR4-mediated inflammation in LN. Globo series GSLs Gb3 and Gb4 are elevated in a mouse model of DN and modulate TLR4-mediated responses in monocytes/macrophages (5). Inhibiting NEU activity may block catabolism of GM3 causing accumulation of HexCer, LacCer and GM3, which may then alter the levels of globo series GSLs and impact TLR4-mediated cytokine expression in MCs.

NEU activity can mediate cell signaling through desialylation of proteins or lipids. With respect to lipids, we previously demonstrated that exogenous addition and cell uptake of GlcCer and LacCer by the immortalized mouse mesangial cell line MES13 stimulates expression of IL-6 and other cytokines without the need for additional stimulants (12). Increasing GlcCer/LacCer levels may be expected to result in elevating sialic acid-containing glycosphingolipids due to the increase in the substrate (LacCer) from which they are synthesized. Recently, we observed levels of ganglioside GM3 are elevated (unpublished observations) in addition to GlcCer and LacCer in the kidneys of nephritic MRL/lpr lupus mice (1). GM3 is the simplest sialic acid-containing ganglioside from which all other gangliosides are generated. Gangliosides present in cell membranes can modulate cell functions by organizing lipid rafts impacting receptor-mediated signal transduction (49). With respect to MCs, increasing GM3 levels inhibits TGF β 1-induced proliferation of rat MCs (50). Thus, a low level of GM3 may contribute to cytokine production in lupus-serum stimulated MCs while extremely high levels, which we observed when NEU activity was inhibited, may reduce cytokine production.

Sialic acid (SA)-containing glycoproteins on the cell surface also play roles in cellular functions. In particular, SAs can mask receptors from ligands to prevent signaling (51) and shown to modulate LPS-mediated TLR4 signaling (44). Our results here suggest removal of SAs by NEU activity mediates lupus serum- and LPS-stimulated TLR4-MAPK signaling. The extracellular domain of TLR4 contains nine N-glycans, which can be sialylated, and binds siglecs and TLR4 activation triggers NEU1 translocation to the cell surface to disrupt TLR4-siglec interactions and allow signaling (43,44,52). With respect to the kidney, NEU activity was shown to mediate LPS-stimulated NF κ B activation in HEK 293T human embryonic kidney cells (44) and sialic acids reduced TLR4 activation and neutralized LPS toxicity induced renal injury *in vivo* (53). Both TLR4 and its co-receptor MD2 are sialylated; however, the N-glycan structures of TLR4 are unknown making it difficult to determine if any of the N-glycans identified in our analysis may be TLR4. Since sialic acid-containing N-glycans were widely impacted when NEU activity was inhibited, there may be receptors/co-receptors in addition to TLR4/MD2 involved in NEU-mediated cytokine release in lupus serum-stimulated MCs, either independent of or in conjunction with TLR4. Future studies are focused on identifying the targets of NEU in modulating IL-6 release through TLR4-MAPK signaling.

The pro-inflammatory activity of lupus serum is largely thought to require the presence of circulating immune complexes. The presence of autoantibodies and immune complexes, with the ability to activate Fc γ Rs and TLRs,

leads to increased production of inflammatory mediators, such as cytokines, chemokines, and reactive oxygen species. Fc γ Rs do not appear to be required in lupus serum-stimulated release of IL-6, GM-CSF or MIP1 α in MRL/lpr MCs. These results agree with previous studies demonstrating that MRL/lpr mice lacking the Fc γ chain still develop renal disease (54) and Fc γ Rs are dispensable for activation of MRL/lpr MCs by pathogenic anti-DNA antibodies (37). Removing IgG complexes from lupus serum using anti-IgG beads did not reduce IL-6 secretion (unpublished data) suggesting lupus serum-stimulated IL-6 secretion by MCs does not require IgG immune complexes either. Interestingly, lupus serum heated to 56°C for 30 min failed to stimulate secretion of IL-6 in MRL/lpr MCs (unpublished data), suggesting complement may play a role. Future studies are aimed at determining if complement in lupus serum are responsible for stimulating NEU-TLR4-MAPK mediated secretion of IL-6 by MCs. Together, these results suggest TLR4- ERK/p38 MAPK signaling pathways play a role in the lupus serum-stimulated release of IL-6. Furthermore, MAPK ERK signaling is necessary for GM-CSF release in response to lupus serum through an unknown receptor.

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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. IL-6 (A), GM-CSF (B), and MIP1 α (C) were measured by individual ELISAs in the media of primary MRL/lpr MCs cultured for 6 hrs in the absence or presence of increasing concentrations of lupus serum. IL-6 (D), GM-CSF (E), and MIP1 α (F) were measured by individual ELISAs in the media of primary MRL/lpr MCs cultured for the indicated time in the absence or presence of 10% lupus serum. IL-6 (G), GM-CSF (H), and MIP1 α (I) mRNA expression were measured by real-time RTPCR in primary MRL/lpr MCs cultured for 6 hrs in the absence or presence of 10% lupus serum. Experiments were performed at least three times with representative results presented.

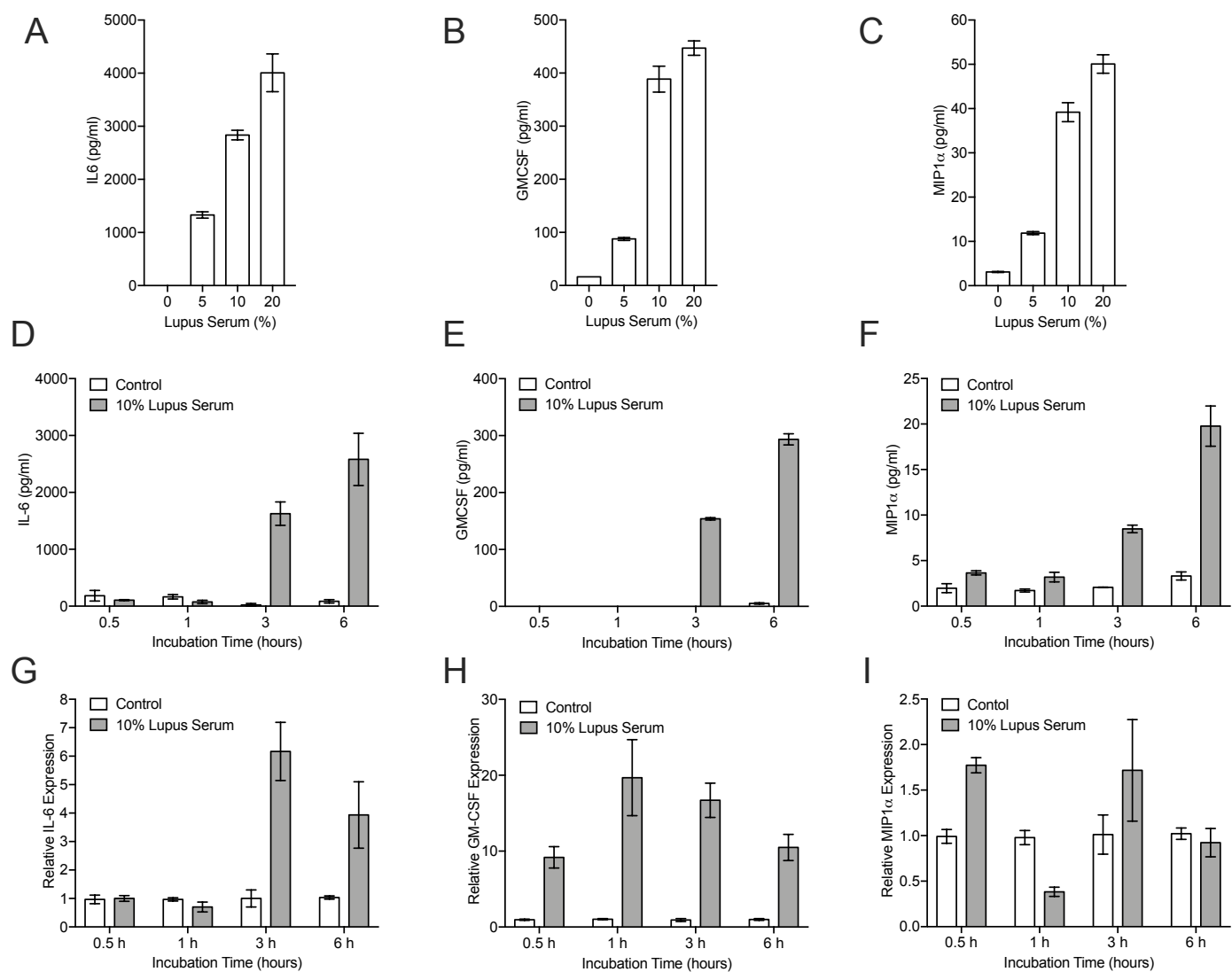


Fig. 2. Inhibiting NEU activity significantly reduces lupus serum-stimulated secretion of IL-6, GM-CSF, and MIP1 α , and expression of IL-6. Primary MRL/lpr MCs were cultured in the absence or presence of 10% lupus serum (LS) and NEU inhibitor oseltamivir phosphate (OP) as indicated in Materials and Methods. IL-6 (A), GM-CSF (B), and MIP1 α (C) were measured by individual ELISAs in the media 6 hrs after addition of LS. IL-6 (D) and GM-CSF (E) mRNA expression were measured by real-time RTPCR in the cells 6 hrs after addition of 10% LS. F) NEU activity was measured in live cells after pretreatment with vehicle (water) or OP followed by stimulation with 10% LS for 6 hrs as indicated in Materials and Methods. NEU activity was measured at the indicated intervals after addition of NEU substrate. Experiments were performed at least three times with representative results presented.

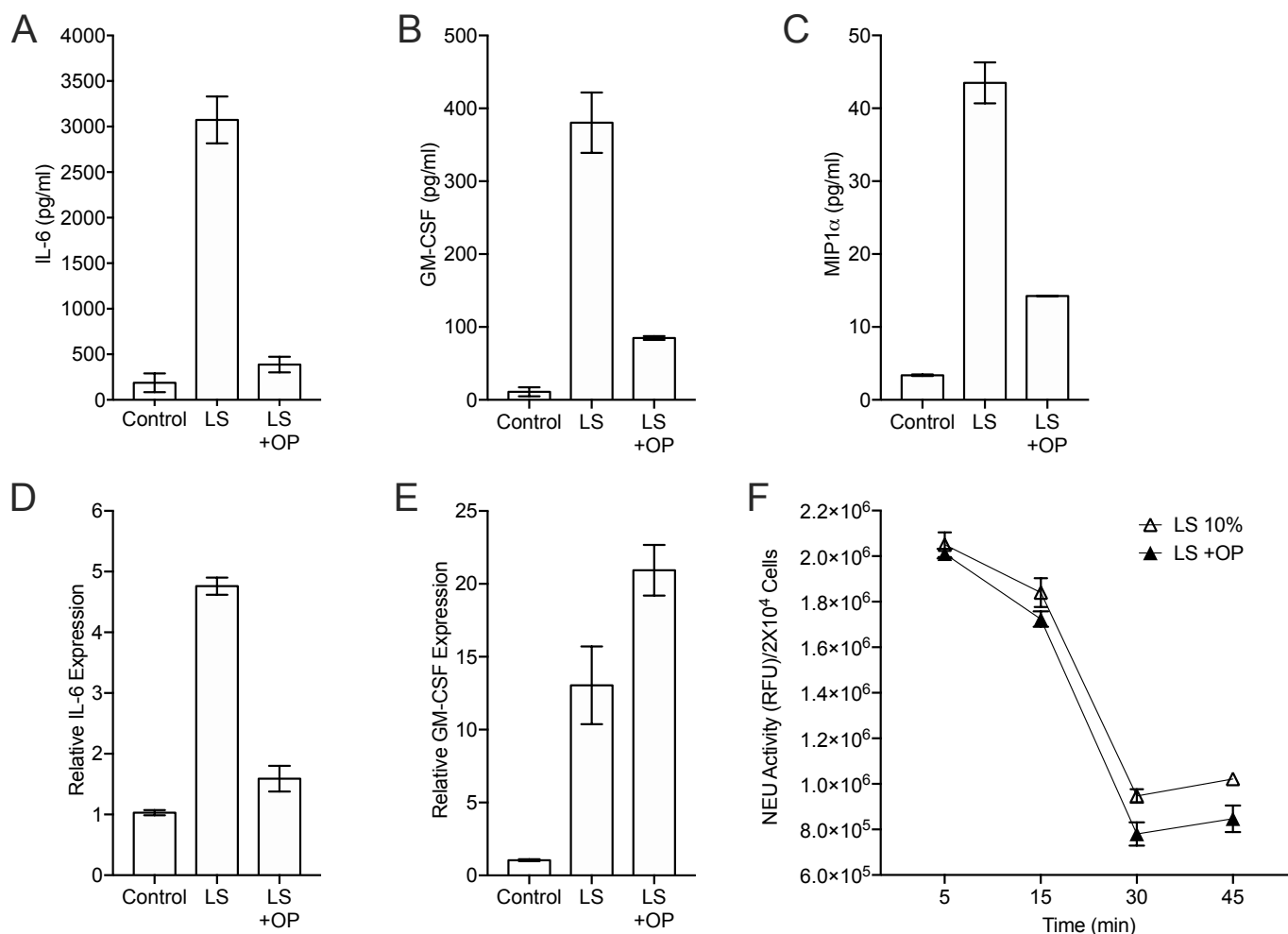


Fig. 3. Lupus serum-stimulated IL-6 secretion is significantly decreased TLR4 is blocked. Primary MRL/lpr MCs were cultured with 10% lupus serum for 6 hrs following pretreatment without or with the indicated antibodies (Ab). IL-6 (A), GM-CSF (B), and MIP1 α (C) were measured in the media by ELISA. Pretreatment with appropriate isotype controls failed to inhibit cytokine secretion (data not shown). Experiments were performed at least three times with representative results presented.

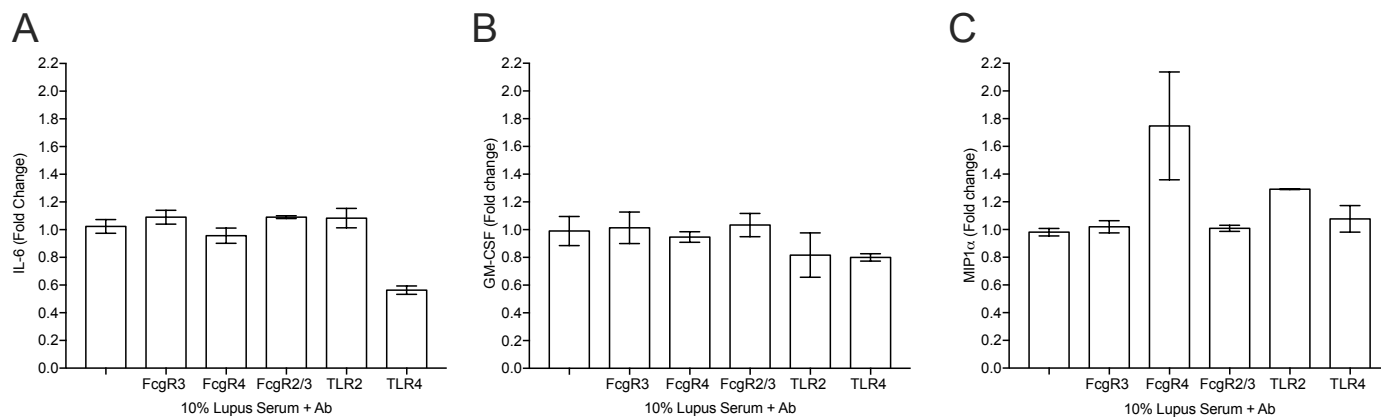


Fig. 4. LPS stimulates expression and secretion of IL-6 and GM-CSF, and secretion of MIP1 α from lupus prone primary MCs. IL-6 (A), GM-CSF (B), and MIP1 α (C) were measured by individual ELISAs in the media of primary MRL/lpr MCs cultured for 6 hrs in the absence or presence of increasing amounts of LPS. IL-6 (D), GM-CSF (E), and MIP1 α (F) were measured by individual ELISAs in the media of primary MRL/lpr MCs cultured for the indicated time in the absence or presence of 5 ng/ml LPS. IL-6 (G) and GM-CSF (H) mRNA expression were measured by real-time RTPCR in primary MRL/lpr MCs cultured for 6 hrs in the absence or presence of 5 ng/ml LPS. Experiments were performed at least three times with representative results presented.

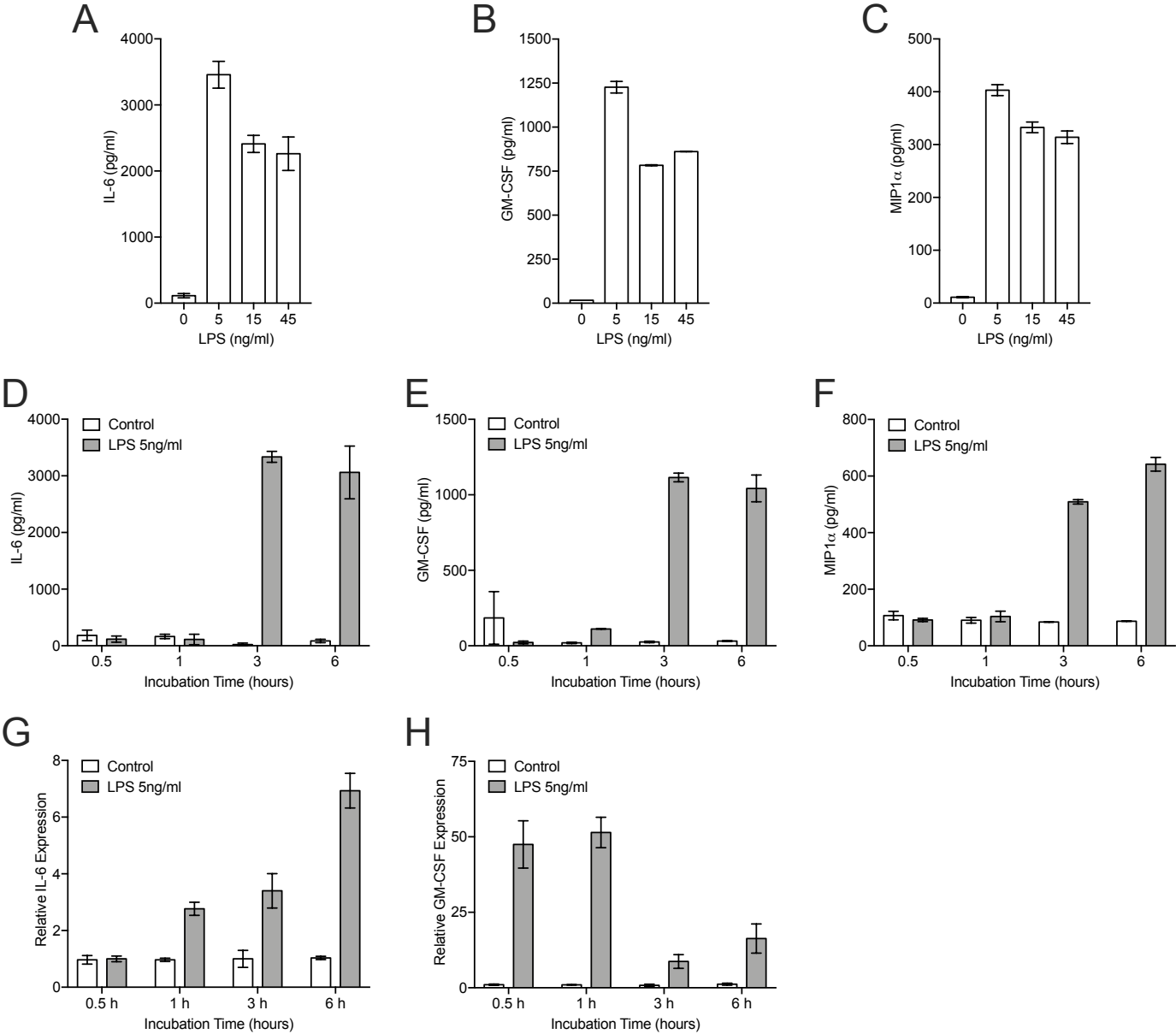


Fig. 5. Inhibiting NEU activity significantly reduces LPS-stimulated secretion of IL-6, GM-CSF, and MIP1 α , and expression of IL-6 and GM-CSF. Primary MRL/lpr MCs were cultured in the absence or presence of 5 ng/ml LPS and NEU inhibitor oseltamivir phosphate (OP) as indicated in Materials and Methods. IL-6 (A), GM-CSF (B), and MIP1 α (C) were measured by individual ELISAs in the media 6 hrs after addition of LPS. IL-6 (D) and GM-CSF (E) mRNA expression were measured by real-time RTPCR in the cells 6 hrs after addition of LPS. F) NEU activity was measured in live cells after pretreatment with vehicle (water) or OP followed by stimulation with 5 ng/ml LPS for 6 hrs as indicated in Materials and Methods. NEU activity was measured at the indicated intervals after addition of NEU substrate. Experiments were performed at least three times with representative results presented.

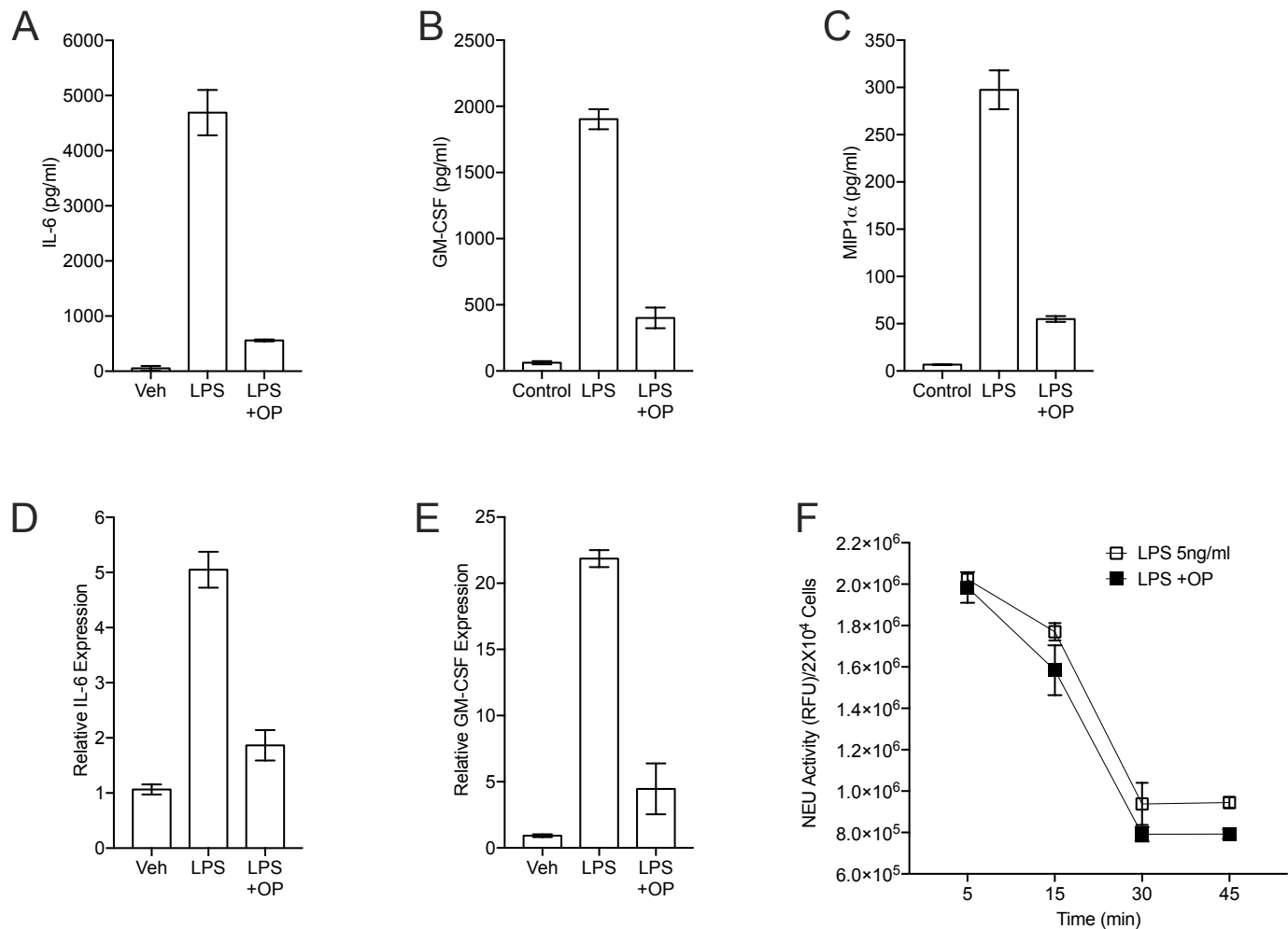


Fig. 6. LPS-stimulated IL-6 and GM-CSF secretion are significantly decreased TLR4 is blocked. Primary MRL/lpr MCs were cultured with 5 ng/ml LPS for 6 hrs following pretreatment without or with the indicated antibodies (Ab). IL-6 (A), GM-CSF (B), and MIP1 α (C) were measured in the media by ELISA. Pretreatment with appropriate isotype controls failed to inhibit cytokine secretion (data not shown). Experiments were performed at least three times with representative results presented.

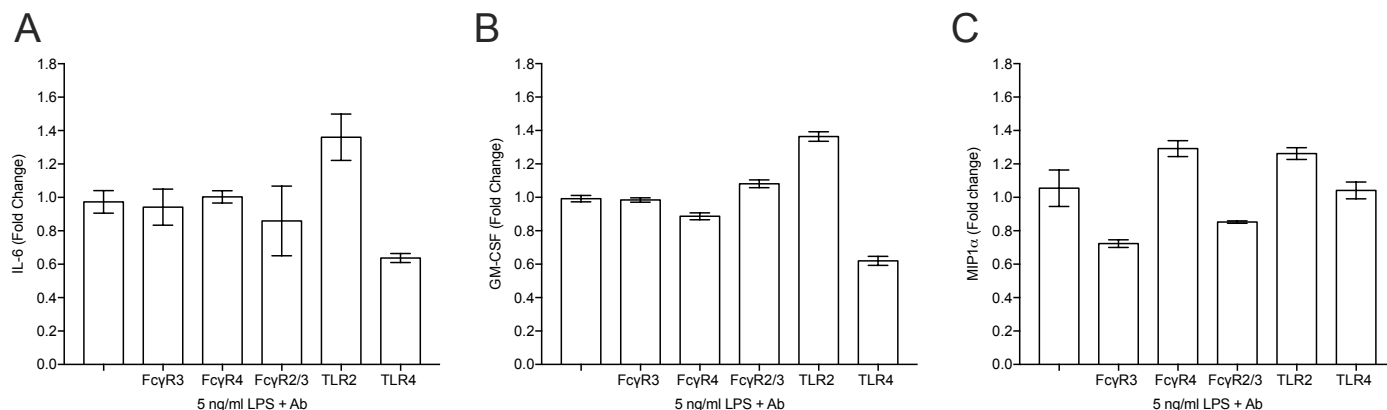


Fig. 7. NEU activity mediates IL-6 and GM-CSF secretion through ERK or p38 MAPK signaling. MRL/lpr primary MCs were stimulated with 10% lupus serum (A, C, D) or 5 ng/ml LPS (B, E, F). Inhibitors for ERK (U0126), p38 (SB203580), JNK (SP600125), or PI3K (Wortmannin) were incubated with the cells prior to addition of 10% lupus serum (A) or 5 ng/ml LPS (B). Cells were pretreated with vehicle (water) or NEU inhibitor oseltamivir phosphate (OP) and stimulated with 10% lupus serum (C and D) or 5 ng/ml LPS (E and F) for the indicated time. Whole cell extracts were prepared and subjected to western immunoblot using antibodies to phosphorylated ERK (P-ERK), total ERK (T-ERK), phosphorylated p38 (P-p38), and total p38 (T-p38). Ratio of P-ERK to T-ERK (C and E) and P-p38 to T-p38 (D and F) are graphed from a representative blot. Experiments were performed at least three times with representative results presented.

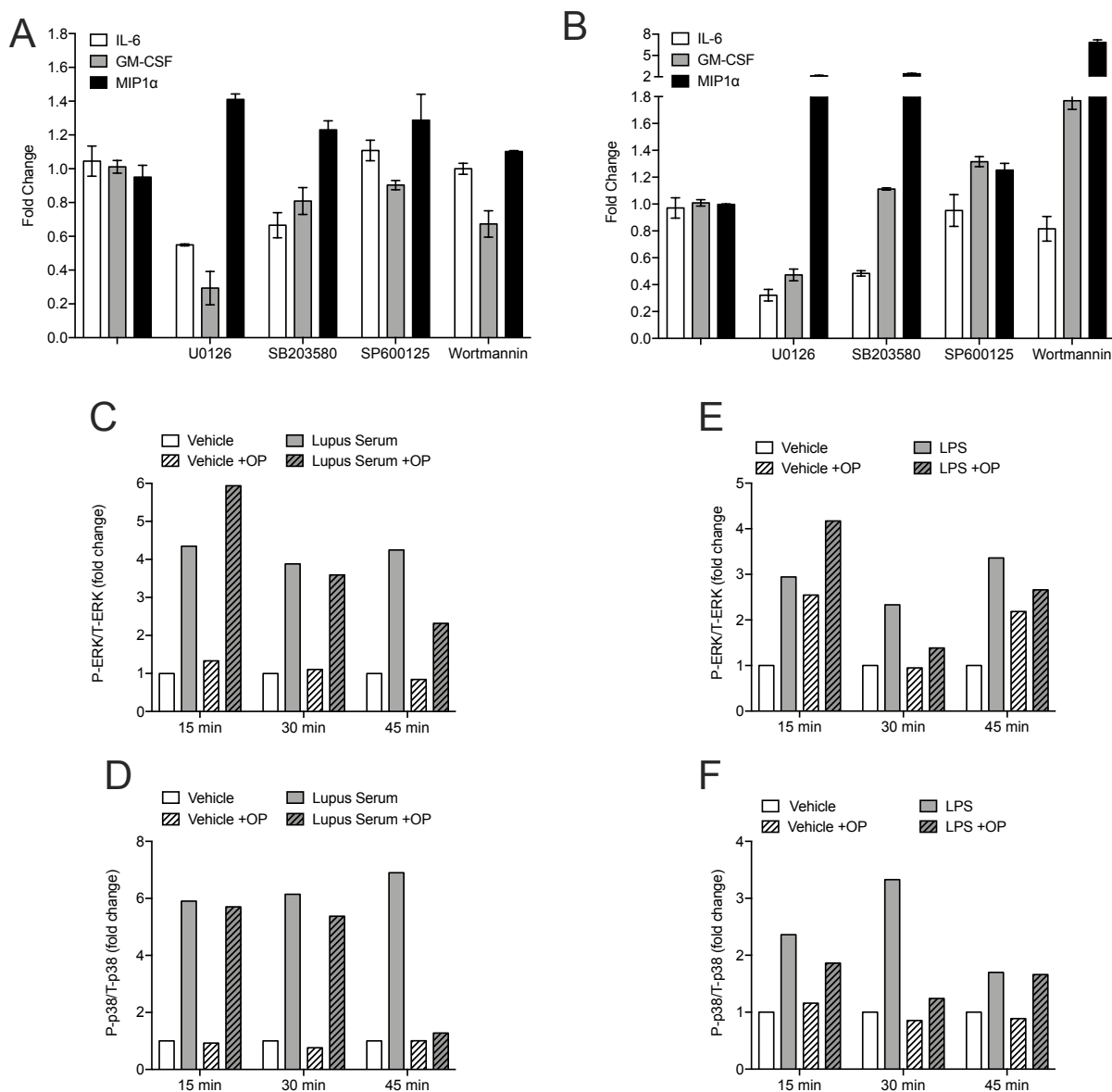
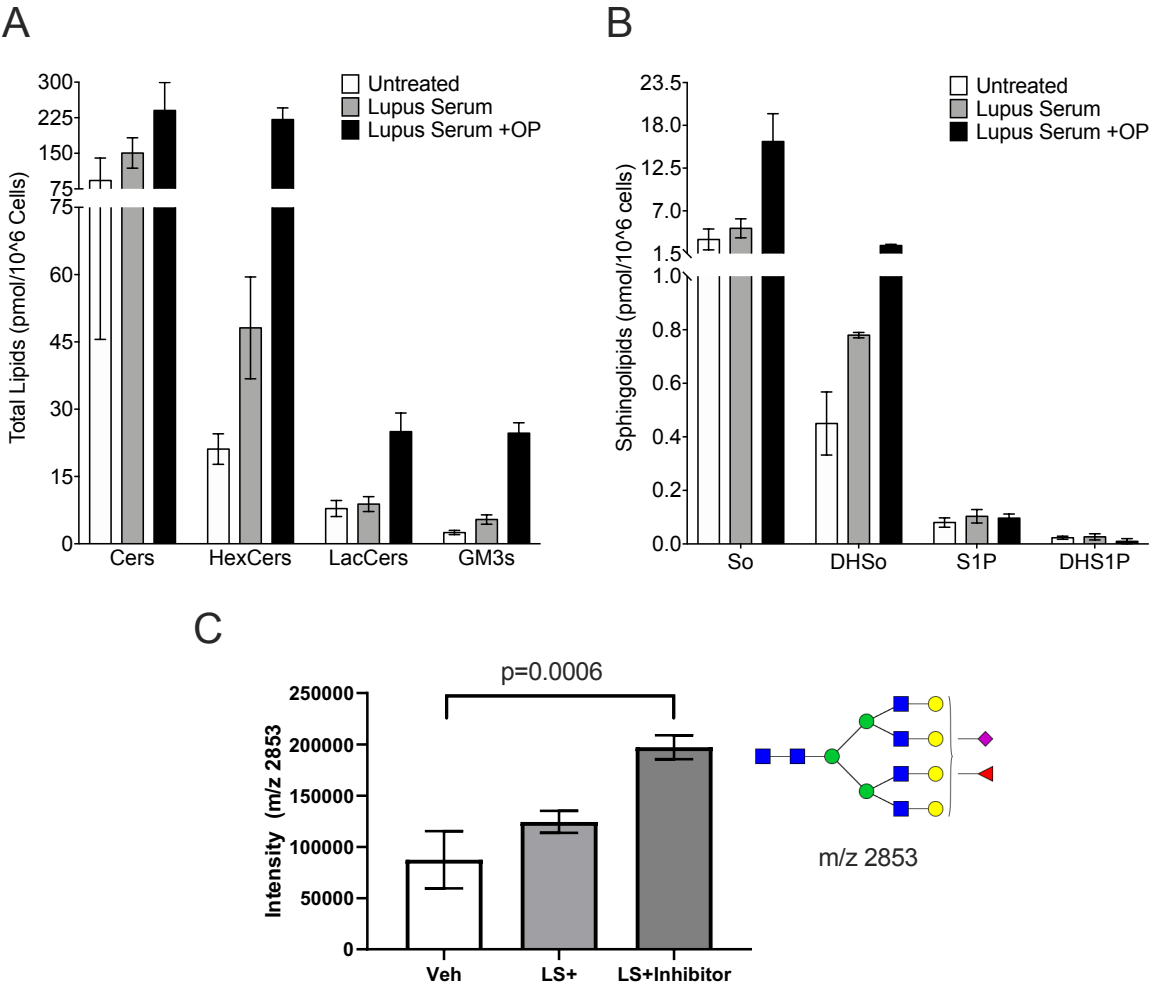


Fig. 8. Glycosphingolipids and sialic acid-containing glycoproteins are significantly increased when NEU activity is blocked in lupus serum-stimulated MRL/lpr primary MCs. MRL/lpr Primary MCs were pretreated with vehicle (water) or the NEU activity inhibitor oseltamivir phosphate (OP) and stimulated with 10% lupus serum for 6 hrs. Untreated, MCs received vehicle, but were not stimulated with lupus serum. Lupus serum, MCs received vehicle and stimulated with 10% lupus serum. Lupus serum +OP, MCs received OP and stimulated with 10% lupus serum. A) Lipid chain lengths C12 to C24 for ceramides (Cers), hexosylceramides (HexCers), lactosylceramides (LacCers), and gangliosides (GM3s) were measured in 10⁶ cells and total of all chain lengths are presented on the graph. B) Sphingolipids sphingosine (So), dihydrosphingosine (DHSo), sphingosine-1 phosphate (S1P), and dihydrosphingosine-1 phosphate (DHS1P) were measured in 10⁶ cells. C) MRL/lpr primary MCs were treated with vehicle (water), lupus serum (LS) +vehicle, or LS +OP (Inhibitor) and N-glycans analyzed by MALDI-FTICR with a representative sialylated N-glycan presented.



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Glycosphingolipid and Proteins in Urine Exosomes: Potential Biomarkers of Lupus Nephritis

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Background/Purpose: Exosomes, extracellular vesicles that are abundant in human urine and contain proteins from renal cells, are a potential source of biomarkers of renal disease in LN patients. We previously demonstrated that levels of Glycosphingolipids (GSL) glucosylceramide (GlcCer) and lactosylceramide (LacCer), and expression of a neuraminidase (NEU1) that mediates GSL catabolism, are elevated in the urine of human patients with nephritis compared to controls. Based on these results we hypothesized that lipids and proteins in the GSL catabolic pathway in urine exosomes could be used as biomarkers of therapeutic response and/or disease flare in LN patients.

Methods: Lipids and proteins were analyzed by mass spectrometry in exosome fractions isolated from urine in 1) a pilot study of lupus nephritis (LN) patients during quiescent disease (non-flare) and renal flare, and in non-nephritis lupus patients; and 2) LN patients that failed to respond or had a complete response to mycophenolate mofetil (MMF) treatment.

Results: In the LN flare versus non-flare versus non-nephritis pilot study, differences in GSL (GlcCers and LacCers) levels were observed among the three sets of samples. Differences in phospholipids were also observed. Proteomics results identified several abundant and known urine biomarkers for LN albumin and serotransferrin in the samples from LN patients. Additional known urine biomarkers in the LN samples included complement factors, anti-trypsin, and alpha-2-HS-glycoprotein/fetuin-A. Novel proteins that were differentially abundant (either higher or lower) in LN samples compared to non-nephritis lupus samples and/or in flare compared to non-flare samples also were identified and included lysosomal protective protein (cathepsin A), a co-factor for NEU1. In the treatment response study, significant differences in GlcCer levels in exosomes isolated from pooled urine samples collected at baseline (prior to MMF treatment) were observed between LN patients that responded compared to those that did not respond to therapy. These results are being validated in a cohort of MMF responder (30) and non-responder (30) LN patients using exosomes isolated from individual urine samples taken at baseline and at 3-, 6-, and 12-months post-treatment. Additional lipids and proteins that are differentially abundant in these urine exosome samples between responders and non-responders are being identified and validated.

Conclusions: Preliminary data suggest that lipid and protein molecules in urine exosomes may serve as early markers of disease flare and/or response to therapy in LN patients and may enhance the predictive power of current models of response to therapy.

Neuraminidase activity mediates IL-6 secretion from activated lupus mesangial cells through TLR4 and MAPK p38

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Glycosphingolipid (GSL) levels and the activity/levels of the enzyme neuraminidase (NEU), which mediates GSL catabolism, are elevated in the kidneys and/or urine of lupus mice and human patients with nephritis compared to their non-nephritic counterparts and healthy controls. We recently demonstrated that NEU activity mediates IL-6 secretion from mesangial cells (MCs) in response to activation by heat aggregated IgG (HA-IgG) and lupus serum. Our previous results demonstrated that NEU1 and NEU3 staining partially overlapped with IgG deposits in cell cultures of primary MCs stimulated with HA-IgG and in renal sections of kidneys from nephritic lupus mice. Both Fc γ receptors (Fc γ Rs) and TLRs can mediate immune complex activation of cells, are expressed by MCs, and were shown to be involved in progression of lupus nephritis. In this study, we examined the role of Fc γ Rs or toll like receptors (TLRs), and downstream signaling pathways in NEU-mediated secretion of IL-6 by activated lupus prone MCs.

Cell surface receptors and MAPK pathways were identified by treating MC cultures with antibodies and inhibitors, respectively, prior to activation with HA-IgG or lupus serum. Phosphorylation of p38 was analyzed by western immunoblot. Cellular localization of NEUs and TLR4 was performed by immunofluorescence. Screening of cytokines secreted by MCs was performed using a 20-plex Luminex cytokine array. Levels of individual cytokines were quantified by ELISA.

Blocking TLR4 significantly inhibited, while blocking Fc γ Rs had no effect on, NEU-mediated IL-6 secretion by activated MCs. Preliminary results show overlap between TLR4 and NEU1 at the plasma membrane of MCs. An inhibitor of the p38 MAPK pathway significantly blocked NEU mediated IL-6 secretion. Phosphorylation of p38 was increased following activation of MCs with HA-IgG or lupus serum and was blocked when NEU activity was inhibited or NEU1 expression was reduced. Although several cytokines were secreted in response to activation by both HA-IgG and lupus serum, only IL-6 and IP-10 (CXCL10) were blocked when NEU activity was inhibited.

Together our results suggest that NEU specifically mediates secretion of IL-6 and IP-10, two cytokines important during early development of lupus nephritis, from activated lupus prone MCs through a TLR4-p38 MAPK pathway.