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TITLE: Glycosphingolipids as Therapeutic Targets and Biomarkers of Lupus Nephritis

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13. SUPPLEMENTARY NOTES

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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1. INTRODUCTION:

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

2. KEYWORDS:

Lupus nephritis, Glycosphingolipid, Biomarkers, Neuraminidase

3. ACCOMPLISHMENTS:

The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction.

What were the major goals of the project?

Major Task 1: Treatment of lupus mice with Oseltamivir. MRL/lpr mice were generated in our mouse colony and aged to 12-13 weeks and treated with water or Oseltamivir Phosphate (OP). 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) have been collected from half of the control mice (MRL/MpJ). Urine, blood, kidneys, and spleens and were collected for all. Analyses have been performed for urine albumin levels (proteinuria), renal pathology scores, glycosphingolipid levels in kidney extracts, and number of activated T cells in the serum for the first half of the mice. The remaining analyses are currently being performed for the first set. The second set of mice are nearing completion of the treatment period (subtask 2) and analyses of collected samples will begin immediately afterwards (subtask 4). The B6.SLE1/2/3 lupus prone mice are developing disease much more slowly in our facility than what was previously reported. After consulting with the investigator that developed this strain, we have determined that it is likely an environmental issue, which is not uncommon. Therefore, we amended our current IACUC protocol to extend the age at which we will begin treatment of the B6.SLE1/2/3 (subtask 3) mice. We anticipate beginning this treatment study in early 2019.

Major Task 2: Genetic knockout of Neu1 in lupus mice. The B6 Neu1 knockout has been backcrossed onto the B6.SLE1/2/3 strain (subtask 2, completed). The B6.SLE1/2/3 study has begun with 15 female B6.SLE1/2/3 $Neu1^{+/+}$ and 15 female B6.SLE1/2/3 $Neu1^{+/-}$ mice from which urine and blood are being collected every other week (subtask 3). The B6.SLE1/2/3 $Neu1^{-/-}$ mice are not being bred and used in this study as planned since the total Neu1 knockout on the parental C57BL/6 (B6) background develops a kidney phenotype, which would confound our analyses. As stated above,

the B6.SLE1/2/3 mice are developing disease much more slowly than originally reported. We are observing only trace amounts of proteinuria in our colony up until approximately 28-34 weeks of age in most mice. Therefore, we will be extending this study to approximately 40-44 weeks of age and will complete this study before the end of the year.
Major Task 3: GSLs as a predictor of response to therapy. Isolation of exosomes (subtask 2) on a first set of 10 "discovery" samples has been completed, initial lipid (subtask 3) results are pending, and proteomics (subtask 4) results have been obtained. Exosome isolation from the remaining urine samples is nearly completed (subtask 3). Validation of lipids and proteins identified in the 10 discovery samples will begin in the next few months on all samples including the 10 discovery samples (subtasks 4-6).
Major Task 4: Association of renal and urine GSLs with renal function. Sectioning of biopsy samples have been completed (subtask 2). Due to equipment repairs, the MALDI-FTICR analyses has been delayed until the end of this year (subtask 2).

Although, we have experienced some delays with the mice and MALDI-FTICR equipment, all Major tasks are expected to be completed by the end of next year.

What was accomplished under these goals?

The major activities completed for the second year of the grant included: 1) Generation of all mice required for the treatment and genetic studies; 2) completion of the treatment regimen of the MRL/lpr mice, collection of samples, and initial analyses of samples; 3) genetic study is in process (mice are ageing and samples are being collected); 4) exosome isolation from urine of the human patients and identification of lipids and proteins in 10 of the samples for validation in those 10 samples and the remaining samples; and 5) sectioning of renal biopsies for the MALDI-FTICR analyses. The specific objectives for this past year were to 1) complete the treatment regimen and collection of samples in both the MRL/lpr and B6.SLE1/2/3 mice and complete the collection of samples in the genetic study so that we are poised for proceeding with sample analyses, 2) complete the exosome isolation and the proteomics and lipid discovery for the human urine exosomes samples so we are poised to complete the validation of identified lipids/proteins in all exosome samples. Most tasks are on-schedule within the proposed timeline in the SOW.

Major Task 1: Preliminary results on the MRL/lpr treatment study of the first half of the mice, five water-treated and eight Oseltamivir Phosphate (OP)-treated, are inconclusive. There is a trend of lower urine albumin (proteinuria) in the OP-treated mice (Fig. 1A), but no differences in renal pathology scores (Fig. 1B), spleen weight or length (Fig. 1C, 1D), or the number of activated T cells (Fig. 1E). Lipidomics results suggest that although the OP-treated mice show a trend of increased ganglioside GM3 (Fig. 2A) as expected (due to inhibiting NEU activity from breaking down gangliosides), the levels of LacCers (Fig. 2B) and HexCers (Fig. 2C) are unchanged and/or slightly increased in the OP-treated mice. We hypothesize that this may be due to continued synthesis of LacCers and HexCers from the precursor ceramide, whose levels are similar between the OP- and water-treated groups (Fig. 2D). Once the samples from the remaining treated mice (nine water-treated, seven OP-treated) are analyzed, there should be enough power to determine any significant differences.

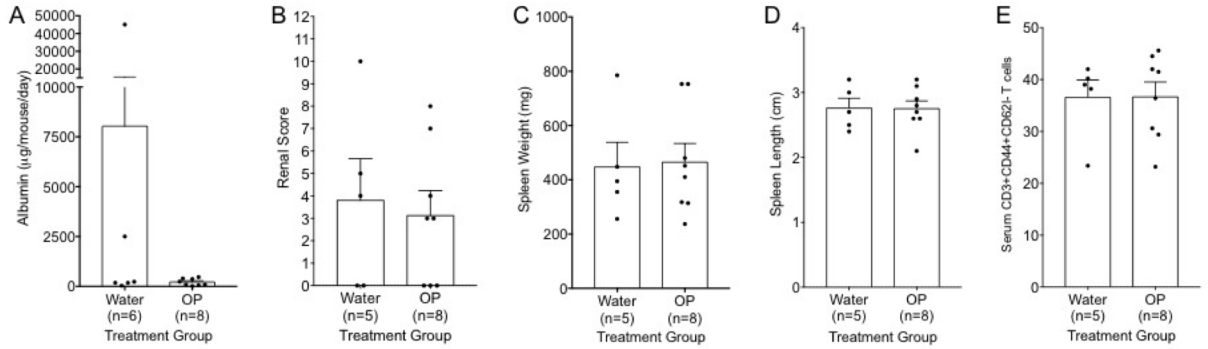

Fig. 1. MRL/lpr female mice were treated with water (n=6) or Oseltamivir Phosphate (OP)(n=8) beginning at 12 weeks of age when at least trace amount of protein was detected in all mice. Mice were randomly assigned a treatment group. Urine and blood were collected just prior to treatment and once a week throughout treatment. Mice were euthanized after four weeks of treatment and kidneys and spleens were collected for analyses. A) Proteinuria was determined by measuring albumin levels by ELISA in 24-hr urine collections. B) One kidney was fixed, sectioned, and stained with H&E and PAS and pathology scored by a pathologist blinded to the treatment. Spleens were weighed (C) and measured (D) before being dissociated into single cell suspensions and used in flow cytometry to quantify the number of activated ($CD44^+$ $CD62L^-$) T cells (E).

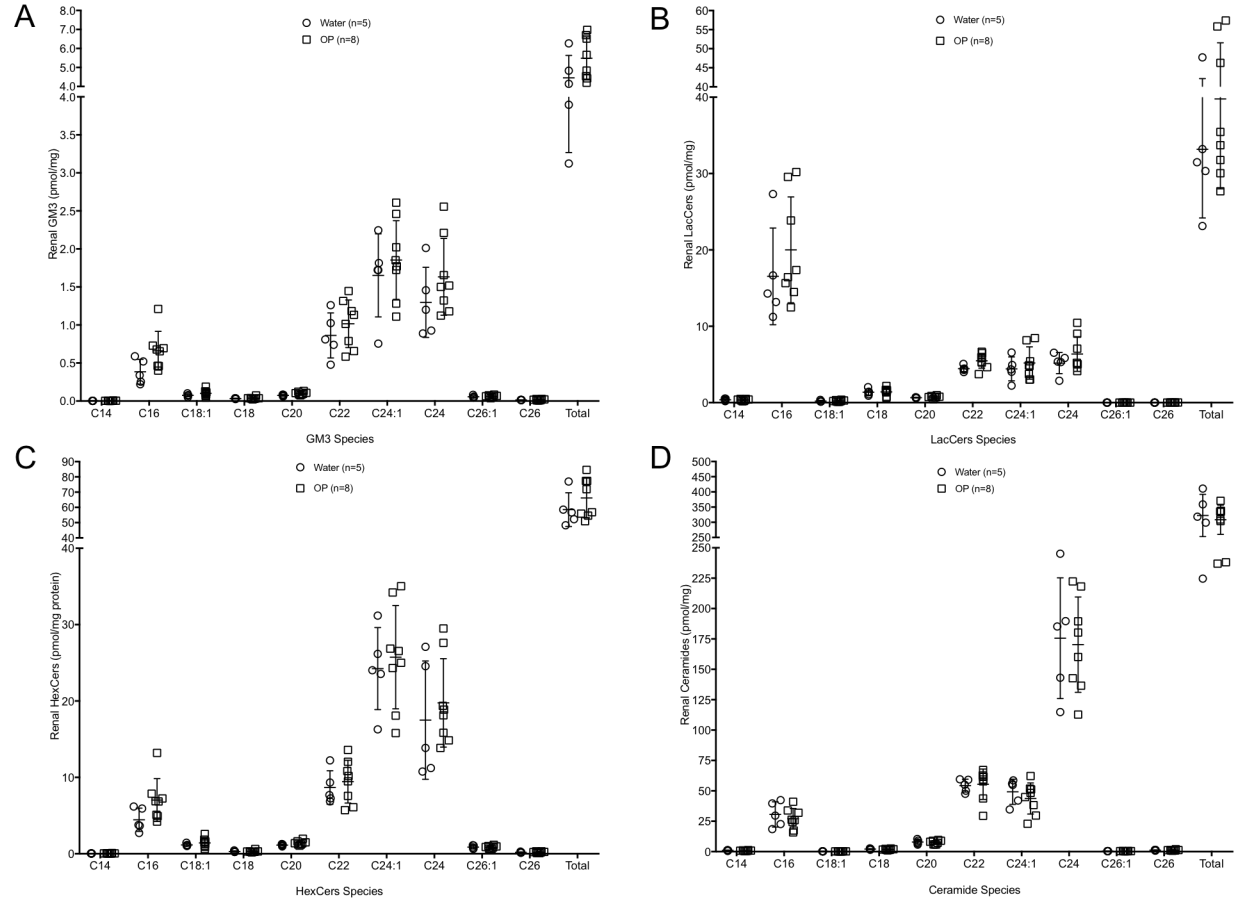

Fig. 2. Kidney homogenates from the same water- and OP-treated mice as in Fig. 1 were analyzed by Mass Spectrometry for changes in glycosphingolipid (GSL) levels of GM3 (A), LacCers (B), HexCers (C), and ceramide (D) species. Individual chain lengths and cumulative of all chain lengths (Total) are shown for each GSL.

Major Task 2: As mentioned above, we determined that the wild-type B6.SLE1/2/3 lupus prone ($Neu1^{+/+}$) mice obtained from Jackson Labs are not developing disease in our animal facility as quickly as previously reported. Hence, urine samples collected from the B6.SLE1/2/3 $Neu1^{+/+}$ and $Neu1^{+/-}$ female mice (15 mice per genotype) have developed only trace or undetectable amounts of proteinuria in both genotypes as of 26 weeks of age (Fig. 3). We are continuing to age them anticipating to end the experiment at approximately 40-44 weeks of age to be able to observe if there are any differences in kidney disease development.

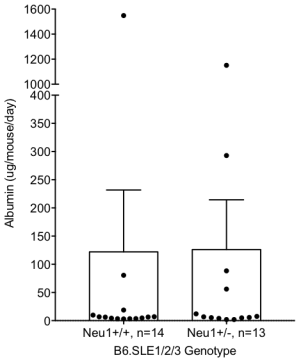

Fig. 3. Urine was collected over a 24-hr period from B6.SLE1/2/3 Neu1+/+ and Neu1+/- mice every two weeks beginning at 18 weeks of age. Urine albumin levels (measure of proteinuria) presented are for all mice that have reached 26 weeks of age.

Major Task 3: Exosomes isolated from urine baseline samples of 10 white female patients with class IV LN, identified as complete responders (CR, n=5) or non-responders (NR, n=5) to mycophenolate mofetil (MMF) treatment 12 months after treatment were used to identify proteins and lipids in discovery proteomics and lipidomics approaches, respectively. Semi-quantitative measures of exosome size and abundance were performed using a Zetaview Exosome Analyzer. Exosome abundance was further quantified using a CD62 ELISA as well as protein assays. Proteomics results (Tables 1-3) of the ten discovery samples detected previously validated and/or identified potential LN urine biomarkers. Two of five previously validated (galectin-3-binding protein, IgG) and six previously identified LN biomarkers (α1-antitrypsin, albumin, haptoglobin, Ig kappa chain C region, serotransferrin, Zn-α-2-glycoprotein) were present in all ten samples (Table 1). Interestingly, another validated LN biomarker, prostaglandin H2 D-isomerase, was detected in four of the five complete responders (CR) and only one of the non-responder (NR) samples (Table 1). An additional eleven proteins (not previously reported) were detected in all ten samples and may therefore, serve as potential novel biomarkers of LN (not shown). Nine proteins were detected in more of the NR samples compared to the CR samples and may serve as potential biomarkers of patients who may fail to respond to MMF treatment (Table 2). The previously identified and potentially novel proteins detected in these samples will be measured in all samples for verification/validation to determine if their presence correlates with a lack of therapeutic response at baseline pre-treatment, 3-month/6-month, and/or 12-month post-treatment.

Previously Validated Urine LN Protein Biomarkers	CR	NR
Galectin-3-binding protein	5/5	5/5
Ig gamma-1 and -2 chain C region	5/5	5/5
Prostaglandin-H2 D-isomerase	4/5	1/5
Previously Identified Urine LN Protein Biomarkers	**CR**	**NR**
Haptoglobin	5/5	5/5
Serum albumin	5/5	5/5
Serotransferrin	5/5	5/5
Alpha-1-antitrypsin	5/5	5/5
Zinc-alpha-2-glycoprotein	5/5	5/5
Ig kappa chain C region	5/5	5/5
Transthyretin	3/5	4/5

Table 1. Proteins previously validated and/or identified as LN urine biomarkers that were detected in exosomes from baseline (prior to treatment) urine samples from ten white female patients with class IV LN. Five were identified as complete responders (CR) and five as non-responders (NR) to MMF treatment 12-months post-treatment.

Protein	CR	NR
Complement C3	0/5	3/5
Complement factor B	1/5	3/5
Complement C4-B	2/5	4/5
Hemopexin (beta-1B-glycoprotein)	1/5	4/5
Lactotransferrin	1/5	4/5
EGF-containing fibulin-like extracellular matrix protein 1	2/5	4/5
Heat shock protein beta-1	0/5	3/5
Secreted Ly-6/uPAR-related protein 1	1/5	4/5
Clusterin	1/5	3/5

Table 2. Proteins detected in more NR than CR urine exosome samples from the same baseline (prior to treatment) samples as in Table 1.

Preliminary results of the MALDI-FTICR mass spectrometry analyses of the same exosomes samples have been completed. Significant effort was spent on optimizing the extraction of lipids and GSL from the urine exosome samples. Several approaches were to use established solvent extraction methods for lipids. Each method was effective for extracting mixtures of phospholipids, sphingomyelin and GSL standards. However, when applied to the urine exosome preparations, results have been inconsistent and variable in regards to what lipid species are detected and signal intensities. Therefore, different approaches were evaluated for urine exosomes and analyzed by MALDI-TOF MS. The most effective method for broad range lipid recovery, including expected sphingomyelin, LacCer, and multiple larger mass cardiolipin and triglyceride species was identified. Spectra on urine exosomes from the 5 complete responders and 5 non-responders were analyzed using the most effective approach. We are now proceeding with correlating detected signal and species to the number of exosomes present and protein concentration.

Major Task 4: Initial MALDI-FITCR analysis of two biopsies was performed prior to the equipment needing repairs (Fig. 4). Once all samples have been imaged, lipid levels will be compared to exosome data and clinical/histological measures.

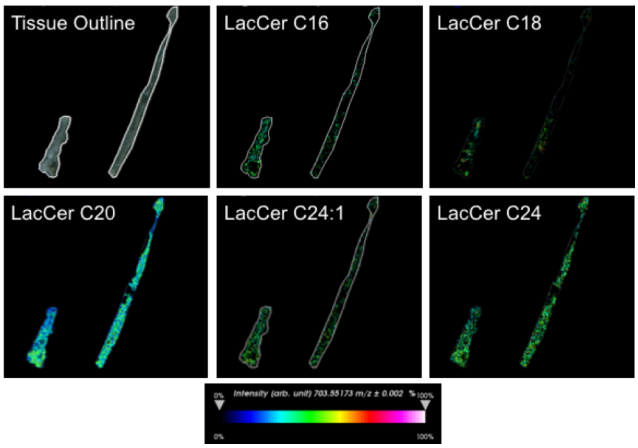

Fig. 4. MALDI-FTICR analysis of LacCers in human renal biopsies from two lupus nephritis patients with class IV nephritis.

Additional achievements stemming from the DoD studies include mechanistic studies that have evolved from working with mesangial cells isolated from kidneys of the DoD study mice. The major findings include demonstrating that IL-6 production upon stimulation of lupus prone mesangial cells with lupus serum occurs in part through a TLR4 (Fig. 5A) and MAPK p38/ERK pathway (Fig. 5B), which is blocked by the NEU inhibitor oseltamivir phosphate (OP) (Fig. 5C). NEU1 may mediate this pathway through direct interaction with TLR4 (Fig. 5D). We further show that NEU inhibitor OP blocks the release of additional cytokines from lupus serum-stimulated (Fig. 6A-C) and LPS-stimulated (Fig. 6D-E) lupus prone mesangial cells. Together these results suggest that NEU mediates mesangial cytokine release in response to inflammatory signals in lupus serum in part through a TLR4-MAPK p38 pathway.

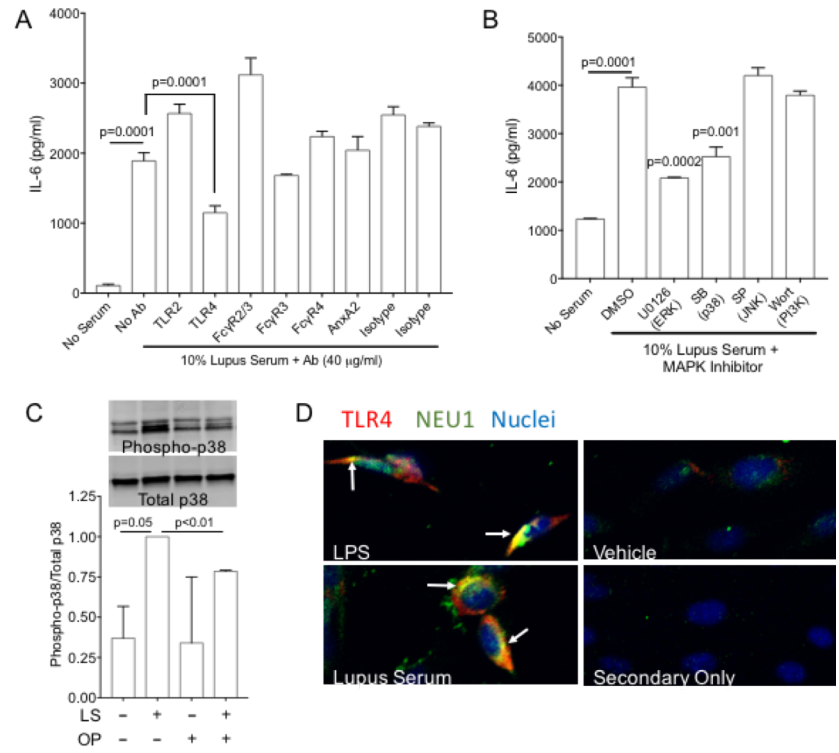

Fig. 5. Lupus prone primary mesangial cells were cultured in the absence or presence of 10% serum from nephritic lupus (lupus serum) mice for 6 hrs. Prior to stimulation, cells were pre-incubated with the indicated antibodies for 2 hrs (A) or MAPK inhibitors (B) and IL-6 was quantified in media by ELISA. C) Prior to stimulation cells were pre-incubated with the NEU inhibitor oseltamivir phosphate (OP) for 24 hrs. Phosphorylation of p38 was analyzed by western immunoblotting. Phospho-p38 and total p38 levels were quantified using ImageJ software and are presented as the ratio of phosphor-to total p38 in the graph (LS, lupus serum). D) Cells were stimulated with lupus serum or TLR4 ligand LPS (or vehicle) and stained for NEU1 (green) and TLR4 (red). Arrows indicate areas of overlap between NEU1 and TLR4 (yellow). Nuclei (blue).

A

	IL-6	IP-10	MIP1α	FGF	IL-17	GM-CSF	IL-5	IL1β	IL-12	IFN-γ	IL-2	IL-13	VEGF	TNFα	MCP-1
B6 Serum	UD	UD	UD	17.26	0.29	101.91	6.41	17.87	1.64	9.21	7.43	27.57	817.12	12.29	1117.83
Lupus Serum	62.70	10.65	150.41	92.30	2.99	252.22	15.27	62.03	44.89	70.74	35.37	18.68	685.86	14.79	1435.46
Lupus Serum +OP	18.30	7.28	104.30	65.14	0.80	95.69	8.82	41.89	43.25	66.29	34.89	5.48	274.65	14.52	988.95

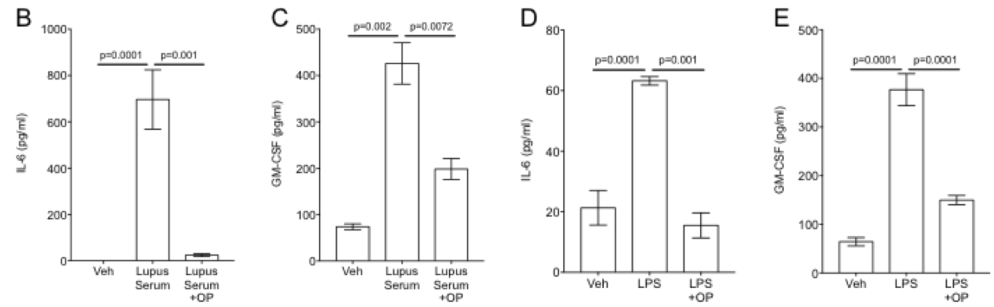

Fig. 6. Lupus prone primary mesangial cells were stimulated with serum from nephritic lupus mice or age-matched non-lupus prone C57BL/6 (B6) mice for 6 hrs following pre-treatment without or with the NEU inhibitor oseltamivir phosphate (OP). A) Multiplex FlexMap3D cytokine analysis was used to measure 15 proinflammatory cytokines in the media. OP blocked 8 cytokines that were increased in response to lupus serum compared to B6 serum (highlighted in blue). An additional 3 cytokines were increased in response to lupus serum, but unaffected by OP (highlighted in yellow), while 4 cytokines were not increased in response to lupus serum compared to B6 serum (highlighted in green). Individual ELISAs confirmed that IL-6 (which we demonstrated previously) (B) and GM-CSF (C) secretion increases in response to lupus serum and are blocked by OP. OP also blocks IL-6 (D) and GM-CSF (E) secretion in response to TLR4 ligand LPS.

All goals are on schedule as described/planned in the SOW and are projected to be completed as indicated.

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

The PI attended the American Society of Nephrology Kidney Week conference in New Orleans, LA and the Southeastern Regional Lipid Conference in Cashiers, N.C. in November, 2017 and presented research findings stemming from the DoD studies. The PI met with collaborators at the two conferences, establishing a new collaboration with Dr. Ron Falk, an expert in pathophysiology of lupus nephritis who graciously agreed to guide us in analyzing GSL levels in patient renal biopsies with respect to clinical disease measures. Additionally, the PI regularly met with the co-investigators of the grant studies approximately every three months to discuss the project, preliminary findings, and experimental strategies.

How were the results disseminated to communities of interest?

The PI presented at the American Society of Nephrology Kidney Week conference in New Orleans, LA and the Southeastern Regional Lipid Conference in Cashiers, N.C. in November, 2017 on the preliminary results provided in the previous progress report and additional *in vitro* oseltamivir treatment of primary mesangial cells, which are supported in part as an extension of the proposed studies that are providing additional insight into the mechanisms by which NEU is mediating inflammation in the LN kidney.

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

Major task 1: The treatment of the MRL/lpr mice (subtask 2) will be completed by early November and the sample analyses (subtask 4) will be completed in early 2019. The treatment of the B6.SLE1/2/3 (subtask 3) will be performed in early 2019 as these mice will need to be aged much longer than anticipated. The treatment period will be followed by analyses of collected samples and expected to be completed by the end of year 3.
Major task 2: Collection of samples is expected to be completed by the end of October, 2018 (subtask 3) and all analyses of samples completed within the next four months (subtask 4).
Major task 3: Isolation of urine exosomes from all remaining patient samples is currently on-going and expected to be completed in the next month (subtask 3). Lipidomics (subtask 4 and 7) and protein (subtasks 5-7) analyses on all samples will be performed once all sample isolations are complete. Candidate proteins and lipids identified in the 10 “discovery” samples will be the focus of these validation analyses.
Major task 4: MALDI-FTICR and data analyses of the renal biopsies are pending a backlog of sample analyses following repair of the MALDI-FTICR equipment (subtask 3) with completion by early 2019.

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?

In addition to generating a global knock down of the neuraminidase 1 (Neu1) gene on the background of the lupus prone strain B6.SLE1/2/3, which will benefit lupus researchers interested in studying the effects of reducing NEU1 in multiple tissues and/or cell types, our results thus far indicate several novel findings. The preliminary OP-treatment study results in the MRL/lpr lupus prone strain suggest that while blocking the glycosphingolipid (GSL) catabolic pathway increases ganglioside GM3 levels LacCers and GlcCers are not reduced as expected. This is likely due to continued synthesis of GlcCer and LacCers from the precursor ceramide. Since a profound impact on proteinuria and renal pathology is not readily apparent, it is possible that the levels of LacCers/GlcCers are more important than gangliosides or both play a role (and the balance is important) in LN disease pathology. Moreover,

these results suggest that blocking both suggest that blocking both the GSL synthesis and catabolic pathways is required to reduce LacCers/GlcCers and increase gangliosides (and balance their levels) to improve disease pathology. Completion of the second set of MRL/lpr OP-treated mice and treatment in the second lupus prone strain B6.SLE1/2/3 will determine significance of our preliminary results.

Preliminary data from the urine exosome analyses identified several known biomarkers of LN, confirming that this is a valid approach to identify potential biomarkers. More importantly, we putatively identified several novel proteins, including a few that may be used to identify patients that are likely to respond or not to mycophenolate mofetil.

What was the impact on other disciplines?

The preliminary results in the OP-treated MRL/lpr lupus mice may be informative for other kidney diseases which exhibit altered GSL metabolism.

Was the impact on technology transfer?

Nothing to report

What was the impact on society beyond science and technology?

Nothing to Report

5. **CHANGES/PROBLEMS:** The Project Director/Principal Investigator (PD/PI) is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer 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

Nothing to Report

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

The B6.SLE1/2/3 lupus prone mice purchased from Jackson Labs has been slower to develop nephritis in our animal facility. We discovered that switching their water source impacted disease development; however, this strain has still been slower to develop proteinuria than expected. Therefore, the time needed to age mice before observing clinical measures of disease before treatment has had to be extended in Major Task 1, subtask 3 (OP treatment study) and the length of time needed to analyze the mice in Major Task 2 (genetic study).

The MALDI-FITCR equipment has needed extensive repairs over the past year, which has delayed the lipid discovery phase of the exosome analyses (Major task 3, subtasks 3 and 4) and renal biopsy analyses (Major task 4). Equipment is now running and samples are in the que for analyses.

Changes that had a significant impact on expenditures

Nothing to Report

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

Significant changes in use or care of human subjects

Nothing to Report

Significant changes in use or care of vertebrate animals.

Due to the delayed disease development in the B6.SLE1/2/3 strain we have had to extend the time before beginning the treatment study and the length of time needed to collect samples in the genetic study for this strain. An amendment approving the extensions for these studies has been approved by both the local IACUC (9-27-18) and by ACURO (10-9-18).

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.

Sundararaj K, Rodgers JI, Subathra M, Siskind LJ, Bruner E, **Nowling TK**. Neuraminidase activity mediates IL-6 production by activated lupus prone mesangial cells. American Journal of Physiology: Renal Physiology, 2018; 314:F630-F642. PMCID: PMC5966761. (acknowledgement of federal support: yes)

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

Nothing to Report

Other publications, conference papers, and presentations.

Tamara Nowling, Kamala Sundararaj and Jessalyn Rodgers. 2017. Neuraminidase Activity Mediates IL-6 Production by Activated Lupus Prone Mesangial Cells. American Society of Nephrology Kidney Week, New Orleans, LA.

Kamala Sundararaj, Jessalyn Rodgers, **Tamara Nowling**. 2017. NEU1-Mediated IL-6 Production by Mesangial Cells. Southeastern Regional Lipid Conference, Cashiers, NC.

Jessalyn Rodgers, Kamala Sundararaj, Richard Drake, Michael Janech, James Oates, **Tamara Nowling**. 2017. Glycosphingolipids as Biomarkers of Lupus Nephritis. Southeastern Regional Lipid Conference, Cashiers, NC.

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

Nothing to Report

- **Technologies or techniques**

Nothing to Report

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

Nothing to Report

- **Other Products**

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: Tamara Nowling, PhD
Project Role: PI
Research Identifier: ORCID ID: 0000-0002-1445-5415
Nearest person month worked: 4.8
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 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 has assisted with exosome analyses, mouse genotyping, renal biopsy sectioning, and has performed the majority of the mechanistic studies- establishing primary mesangial cell lines, culturing and performing all in vitro oseltamivir treatment studies and analyses of 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 initial renal biopsies and the 10 "discovery" exosome samples analyzing glycosphingolipid expression and levels.

Name: Mike Janech, PhD
Project Role: Co-investigator
Nearest person month worked: 0.6
Contribution to Project: Dr. Janech assisted with the proteomic analyses of the 10 discovery exosome samples.

Name: Jim Oates, MD
Project Role: Co-investigator
Nearest person month worked: 0.24
Contribution to Project: Dr. Oates continues to provide guidance with the human sample analyses and assisted with analysis of the data obtained from the proteomics results of the 10 "discovery" samples.

Name: Beth Wolf, PhD
Project Role: Co-investigator
Nearest person month worked: 0.6
Contribution to Project: Dr. Wolf has assisted with analyzing the mesangial cell data and the preliminary data obtained from the 10 discovery exosome samples and MRL/lpr OP-treatment study.

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

Tamara K. Nowling, PhD: No changes.
James C. Oates, MD: In the past year, Dr. Oates is providing effort on one new study:
NIH/NIAMS AR071947 (05/27/2018-04/30/2023), Predictive biomarkers for disease activity and organ damage in patients with lupus (no overlap with DoD studies). This project is a literature-based candidate approach to identify serum, urine, and genetic biomarkers to develop predictive models of lupus nephritis fare and damage.
Role on project: co-PI, 1.2 CM
Bethany Wolf, PhD: No changes.
Michael Janech, PhD: Dr. Janech recently left the university and no longer have covered salary effort on the grant. He moved to the College of Charleston and is still physically located in the same city and will continue to provide guidance with the proteomics analyses. All remaining proteomics analyses are being completed by the Mass Spectrometry facility located on campus on a fee-for-use basis.
Richard Drake, PhD: In the past year, Dr. Drake had effort end on two projects: R21 CA207779-01, R21 CA186799-01. *He is now providing effort on three new studies (No overlap with DoD studies):*

1) *DoD/PCRP Idea Synergy W81XWH-17-1-0643 (9/30/17-9/30/20), A novel serum and tissue immunoglycomic biomarker panel to distinguish progressive PCa. The goals are to combine immune and glycan molecular markers from prostate tissue and serum to improve prediction of biochemical recurrence and/or variant neuroendocrine-like progressive prostate cancers.*
   Role on Project: Co-PI, 1.8 CM
2) *NIH/CI R01 CA212409 (4/2018-3/2023), Tumor immune and glycan biomarkers for progressive prostate cancer. The goals are to determine the sensitivity and specificity of tissue MIC and serum sMIC and tissue/serum glycan panels in predicting biochemical recurrence in a large cohort of Gleason 6 and 7 prostate cancer samples.*
   Role on Project: Co-PI, 1.8 CM
3) *NIH/NCI Innovative Molecular Analysis Technologies (5/1/18-4/30/20), Glyco-typer: an antibody capture glycan imaging methodology. The goal is to develop a novel method of solid-phase, quantitative N-linked glycan analysis of glycoproteins from tissues and biofluids.*
   Role on Project: Co-I, 0.6 CM

What other organizations were involved as partners?

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: N/A

QUAD CHARTS: N/A

9. APPENDICES:

- Sundararaj K, Rodgers JI, Subathra M, Siskind LJ, Bruner E, **Nowling TK**. Neuraminidase activity mediates IL-6 production by activated lupus prone mesangial cells. American Journal of Physiology: Renal Physiology, 2018; 314:F630-F642. PMCID: PMC5966761.
- **Tamara Nowling**, Kamala Sundararaj and Jessalyn Rodgers. 2017. Neuraminidase Activity Mediates IL-6 Production by Activated Lupus Prone Mesangial Cells. American Society of Nephrology Kidney Week, New Orleans, LA (abstract, poster presentation).
- Kamala Sundararaj, Jessalyn Rodgers, **Tamara Nowling**. 2017. NEU1-Mediated IL-6 Production by Mesangial Cells. Southeastern Regional Lipid Conference, Cashiers, NC (abstract, poster presentation).
- Jessalyn Rodgers, Kamala Sundararaj, Richard Drake, Michael Janech, James Oates, **Tamara Nowling**. 2017. Glycosphingolipids as Biomarkers of Lupus Nephritis. Southeastern Regional Lipid Conference, Cashiers, NC (abstract, podium talk).

CONTROL ID: 2788728

TITLE: Neuraminidase Activity Mediates IL-6 Production by Activated Lupus Prone Mesangial Cells

AUTHORS: Tamara Nowling[1], Kamala Sundararaj[1], Jessalyn I. Rodgers[1]

INSTITUTIONS:

1. Medical University of South Carolina, Charleston, SC, United States.

GROUP OR TEAM (if applicable):

BODY-Background: Glycosphingolipid (GSL) levels and neuraminidase (NEU) (an enzyme that mediates GSL catabolism) activity/expression are altered in the kidneys and/or urine of lupus mice and human patients with proliferative nephritis compared to their non-nephritic counterparts and healthy controls. Specifically, elevated GSL levels were observed in the mesangial region of glomeruli. We hypothesize that activation of mesangial cells (MCs) in the progression of lupus nephritis is mediated in part by NEU activity, contributing to renal inflammation in lupus nephritis. Here we investigated the role and possible mechanisms by which NEU activity contributes to MC activation.

BODY-Methods: For these studies, we used the MES13 mouse MC line and primary MCs grown out from glomeruli isolated from MRL/lpr lupus prone mice. MCs were analyzed in the absence or presence of heat aggregated IgG (mimic of immune complex deposition), inhibitors for NEU activity or MAP kinase pathways inhibitors include real-time RTPCR, NEU activity assays, IL-6 and MCP-1 ELISAs, immunohistochemistry of renal sections, and confocal immunofluorescence of MCs.

BODY-Results: While HA-IgG alone fails to activate MES13 cells to produce IL-6, over-expressing NEU1 or NEU3 alone results in significant production of IL-6. Addition of HA-IgG to MES13 cells over-expressing NEU1 or NEU3 further increased IL-6 production over NEU1 or NEU3 alone. In primary MCs, Neu1 message levels, NEU activity, and IL-6 and MCP-1 production are significantly increased following addition of HA-IgG in a dose-dependent manner. Addition of an FDA-approved inhibitor of NEU activity significantly and dose-dependently inhibited HA-IgG-induced IL-6 while higher concentrations were required to inhibit MCP-1 production. NEU1 and NEU3 appear to co-localize with HA-IgG at the surface of the MES13 and primary MCs. JNK and p38 MAP kinase inhibitors prevented IL-6 production in response to NEU1 or NEU3 over-expression in MES13 cells.

BODY-Conclusion: Together these results suggest that immune complex activated IL-6 production of MCs is mediated by NEU activity. This may occur at the cell surface in a complex of HA-IgG and surface receptor that recognizes HA-IgG. Furthermore, the NEU1/NEU3 mediated IL-6 production appears to involve the p38/JNK stress-activated MAPK pathways. Targeting NEU activity may reduce MC cytokine production and thus renal inflammation in lupus nephritis.

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TABLE TITLE: (No Tables)

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TABLE FOOTER: (No Tables)

Glycosphingolipids as Biomarkers of Lupus Nephritis

Authors: [1]Jessalyn Rodgers, [1]Kamala Sundararaj, [2]Richard Drake, [1]Michael Janech, [1]James Oates, and [1]Tamara Nowling

Authors' Affiliation: [1]Department of Medicine and [2]Department of Pharmacology, Medical University of South Carolina, Charleston, SC

Glycosphingolipid (GSL) levels and activity/expression of neuraminidase (NEU), which mediates GSL catabolism, are elevated in the kidneys and/or urine of lupus mice and human patients with nephritis compared to controls. We also demonstrated that urinary levels of lactosylceramides (LacCers) in lupus mice were significantly elevated prior to significant elevation of proteinuria in the same mice. Preliminary results measuring GSL levels by MALDI-FTICR in exosome fractions from lupus nephritis (LN) patient urine showed significant differences between patients that responded to standard therapy after one year compared to those that did not respond. Exosomes, 20-100 nm extracellular vesicles derived from late endosomes in cells, are abundant in human urine and contain proteins from renal cells and hence, are a potential source of biomarkers of renal disease in LN patients. These data suggest that molecules in this pathway may serve as early markers in LN. We have collected exosomes from LN patient urine samples taken during quiescent disease and during a disease flare. The exosomes were used: 1) to measure GSL levels by MALD-FTICR, 2) to measure NEU1, NEU3, and exosome marker levels by western immunoblot, and 3) for proteomic discovery analysis. In addition, we have generated a NEU1 heterozygote on the B6.SLE1/2/3 lupus prone mouse strain to analyze the effects of decreasing NEU1 levels (and GSL catabolism) on the progression of LN.

Glycosphingolipid Catabolism Mediates Mesangial Cell IL-6 production

Authors: Kamala Sundararaj, Jessalyn Rodgers, and Tamara Nowling

Authors' Affiliation: Department of Medicine Medical University of South Carolina, Charleston, SC

Glycosphingolipid (GSL) levels and neuraminidase (NEU) (an enzyme that mediates GSL catabolism) activity/expression are elevated in the kidneys and/or urine of lupus mice and human patients with proliferative nephritis compared to their non-nephritic counterparts and healthy controls. Elevated renal GSL catabolism suggests this pathway may play a role in mediating the pathogenesis of lupus nephritis (LN). We demonstrated elevation of NEU1 and NEU3 in mesangial cells (MCs) of kidneys in two different strains of lupus mice. Using primary MCs from lupus prone MRL/lpr mice, we demonstrated significant and dose-dependent increases in IL-6 production following stimulation with the immune complex mimic heat aggregated IgG (HA-IgG) and lupus serum. This IL-6 production, which parallels a significant increase in NEU activity, is blocked by the NEU inhibitor oseltamivir phosphate (OP). Compared to the lupus prone MCs, C57BL/6-derived MCs produced significantly increased, but several fold lower, levels of IL-6 in response to HA-IgG and lupus serum. Preliminary data show that NEU activity and IL-6 production were not significantly increased in MCs derived from C57BL/6 $Neu1^{+/-}$ mice compared to MCs derived from C57BL/6 $Neu1^{+/+}$ MCs. Further evidence that NEU activity mediates IL-6 production by MCs was observed in the immortalized MES 13 MC line. Overexpressing NEU1 or NEU3 in MES 13 MCs resulted in a significant dose-dependent increase in IL-6 production in the absence of stimulation. This IL-6 response was blocked by MAPK inhibitors suggesting NEU mediates IL-6 production through MAPK signaling. Co-localization studies detected overlapping expression of NEU1 and NEU3 with HA-IgG on the plasma membrane of primary MCs and with IgG deposits in renal sections of lupus mice. Together, these results suggest that increased GSL catabolism (NEU activity) mediates MC production of IL-6 possibly by interacting with an IgG-receptor complex that triggers MAPK signaling. Targeting the GSL catabolic pathway (NEU activity or specifically NEU1/NEU3), may reduce renal cytokine production and inflammation in lupus nephritis.

Am J Physiol Renal Physiol 314: F630–F642, 2018.
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RESEARCH ARTICLE

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

Kamala Sundararaj,[1] Jessalyn I. Rodgers,[1] Subathra Marimuthu,[3] Leah J. Siskind,[3] Evelyn Bruner,[2] and Tamara K. Nowling[1]

[1]*Division of Rheumatology and Immunology, Department of Medicine, Medical University of South Carolina, Charleston, South Carolina;* [2]*Division of Pathology and Laboratory Medicine, Department of Medicine, Medical University of South Carolina, Charleston, South Carolina; and* [3]*Department of Pharmacology and Toxicology, James Graham Brown Cancer Center, University of Louisville, Louisville, Kentucky*

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

IL-6; lupus nephritis; mesangial cells; neuraminidase

INTRODUCTION

Lupus is a systemic autoimmune disorder that arises with immune system malfunction affecting multiple organs and tissues. Disease is characterized by autoantibody production and immune complex deposition in target organs, leading to inflammation and organ damage. Though lupus affects skin, joints, heart, lungs, and brain, 30–60% of patients develop glomerulonephritis, a leading cause of morbidity and mortality. One of the earliest hallmarks of lupus nephritis (LN) is the deposition of immune complexes in the glomeruli, which leads to production of proinflammatory cytokines and chemokines by resident renal cells, immune cell infiltration, inflammation, and eventually tissue damage (reviewed in Refs. 32 and 55). Despite understanding this general sequence of events that leads to renal damage in LN, the specific molecular mechanisms involved have not been completely elucidated, especially with respect to resident renal cell responses that contribute to the progression of disease. Identifying molecular mechanisms that play a role in renal inflammation and damage will support development of more effective therapeutic approaches for treating lupus patients with nephritis.

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

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

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

MATERIALS AND METHODS

Mice. MRL/lpr and NZM2410 lupus mice between 8 and 34 wk of age (as indicated in the RESULTS) were used for all experiments. Both male and female NZM2410 and MRL/lpr mice were used for analyses in Fig. 1. The sex of mice used to generate primary MRL/lpr mesangial cell lines is described below. C57BL/6 mice (14–16 wk of age) were used as a source of healthy serum. Mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and were maintained on a 12:12-h light-dark cycle with access to food and water ad libitum. All mice were housed under pathogen-free conditions at the animal facility of the Ralph H. Johnson Veterans Affairs Medical Center (Charleston, SC), and all animal experiments were approved by the Institutional Animal Care and Use Committee. Urine protein levels were determined using Chemstrip 7 (Roche Diagnostics, Indianapolis, IN).

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

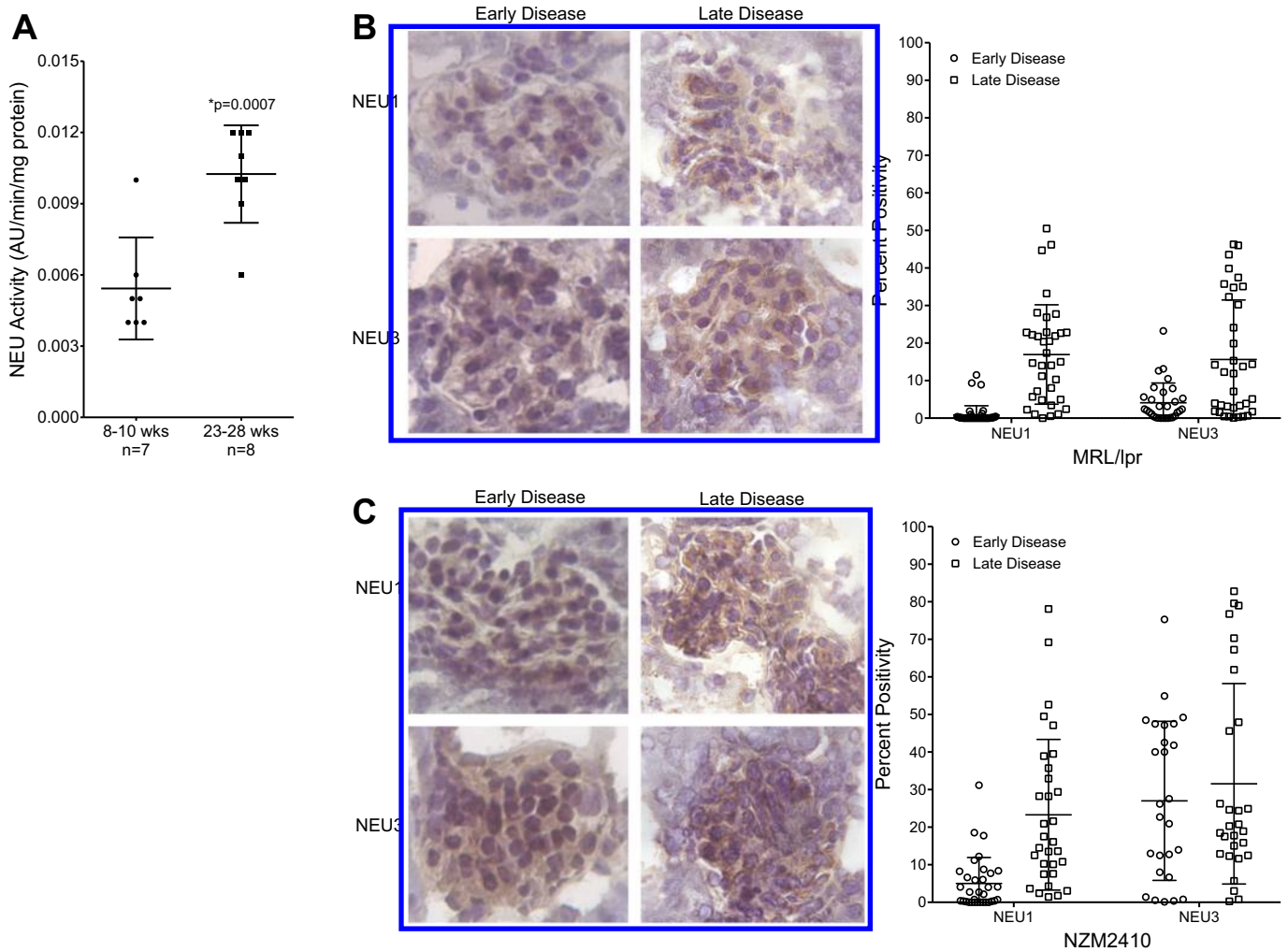

Fig. 1. Renal NEU activity is elevated and NEU1 and NEU3 are highly expressed in MCs of nephritic lupus mice. *A*: NEU activity was measured in kidney homogenates from 8- to 10-wk-old prenephritic and 23- to 28-wk-old nephritic NZM2410 mice. *P* value was calculated as described in MATERIALS AND METHODS.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

fully removed with most of the media enriching the glomeruli to ~95% purity. The glomeruli were cultured at 37°C in a 5% CO_2 incubator in DMEM medium containing 10% FBS, 1% penicillin/streptomycin and ITS supplement (insulin 5 μg/ml, transferrin 5 μg/ml, and sodium selenite 5 ng/ml; Sigma-Aldrich, St. Louis, MO) for ~4 wk with medium changed every 4 or 5 days. After 3 wk, medium was supplemented with D-valine to inhibit fibroblast growth. Once stellate-shaped cells were observed, characteristic of MCs, they were trypsinized with trypsin/EDTA (Sigma-Aldrich) and grown in MEM/D-valine medium (GenDEPOT, Houston, TX).

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

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

Culturing, treatment, and transfection of MES13 mouse mesangial cell line. MES13 mouse mesangial cells (American Type Culture Collection, Manassas, VA) were cultured in low-glucose DMEM: Ham's F12 (3:1) medium supplemented with 5% FBS and 1% penicillin and streptomycin. For treatment of mesangial cells with glycosphingolipids, 0.5×10^6 cells were plated in 10 cm^2 dishes in complete growth media. Forty-eight hours after plating, the media were replaced with complete media containing 0.1 μM glycosphingolipids (an equal mixture of C_8-glucosylceramide and C_8-lactosylceramide, reconstituted in DMSO). After 1 h, the cells were washed with PBS and harvested by scraping in 700 μl of RLT buffer (Qiagen RNeasy mini kit, according to the manufacturer's instructions) for RNA isolation and real-time PCR, as described below. In all cases, an equal volume of DMSO was used as a vehicle control.

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

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

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

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

Immunohistochemistry. Kidneys were removed from MRL/lpr mice at early (6–8 wk old) and late (16–18 wk old) disease stages, and from NZM2410 mice at early (8–10 wk old) and late (28–34 wk old) disease stages. The flash-frozen kidneys were embedded in OCT and sectioned at 4- or 5-μm thickness using a cryotome cryostat. Tissue sections were fixed in ice-cold acetone and blocked in 5% BSA. Sections were incubated with rabbit anti-NEU1 or rabbit anti-NEU3 primary antibodies (Santa Cruz Biotechnology). These antibodies were previously shown to specifically detect NEU1 and NEU3, respectively (10), which we confirmed and further determined do not cross-react in a Western immunoblot of extracts from cells transfected with NEU1 and NEU3 expression vectors (data not shown). Sections were incubated with 0.3% H_2O_2 solution in PBS at room temperature to block endogenous peroxidase activity. After washing in PBS, the sections were incubated with HRP-conjugated goat anti-rabbit secondary antibody (Invitrogen, Carlsbad, CA). DAB substrate solution (Vector Laboratories, Burlingame, CA) was applied on tissue sections for 10 min and counterstained in Carazzi's hematoxylin for 45 s. Tissues were dehydrated and cleared in ethanol gradients and xylene and mounted with Cytoseal 60 (Thermo Fisher Scientific, Carlsbad, CA). Staining with secondary alone was used as a negative control. Images were captured on a Nikon Eclipse 90i microscope with a Nikon Digital Sight D5-Fi1 camera and NIS-Elements AR 3.2 software at ×20–40. NEU1 and NEU3 staining within 8–12 glomeruli per section of three mice for each strain at each disease stage were evaluated using the publicly available plugin IHC profiler (50) for scoring DAB staining intensity using ImageJ 1.51m9 software (41). Data presented are the percentage of staining scored as high positive and positive for each glomerulus.

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

Primer	Sequence
αSMA forward	5′-CCC TGA AGA GCA TCC GAC AC-3′
αSMA reverse	5′-GCC TTA GGG TTC AGT GGT GC-3′
Pdgfβ forward	5′-GTG GTG AAC TTC CAA TGG ACG-3′
Pdgfβ reverse	5′-GTC TGT CAC TGG CTC CAC CAG-3′
Cd31 forward	5′-TAC TGC AGG CAT CGG CAA A-3′
Cd31 reverse	5′-GCA TTT CGC ACA CCT GGA T-3′
Cd144 forward	5′-CAG TGA CAG AGG CCA ATT CT-3′
Cd144 reverse	5′-GCC TCC ACA GTC AGG TTA TAC-3′
Nephrin forward	5′-ACA CAA GAA GCT CCA CGG TTA G-3′
Nephrin reverse	5′-TGG CGA TAT GAC ACC TCT TCC-3′
Neu1 forward	5′-ACG ATG TAG ACA CAG GGA TAG TG-3′
Neu1 reverse	5′-GTC GTC CTT ACT CCA AAC CAA C-3′
Neu3 forward	5′-TTC CAC CTT CCC TTC CTC ATC C-3′
Neu3 reverse	5′-GCA ATA AGC ACC GTT ATC AAC CAT C-3′
βactin forward	5′-AGA TTA CTG CTC TGG CTC CTA G-3′
βactin reverse	5′-CCT GCT TGC TGA TCC ACA TC-3′
Cxcl3 forward	5′-TGT ACC ATG ACA CTC TGC AAC-3′
Cxcl3 reverse	5′-CAA CGA TGA ATT GGC GTG GAA-3′
Cxcl11 forward	5′-GGC TTC CTT ATG TTC AAA CAG GG-3′
Cxcl11 reverse	5′-GCC GTT ACT CGG GTA AAT TAC A-3′
Ifng forward	5′-ATG AAC GCT ACA CAC TGC ATC-3′
Ifng reverse	5′-CCA TCC TTT TGC CAG TTC CTC-3′
Il1a forward	5′-GCA CCT TAC ACC TAC CAG AGT-3′
Il1a reverse	5′-AAA CTT CTG CCT GAC GAG CTT-3′
Il1b forward	5′-GCA ACT GTT CCT GAA CTC AACT-3′
Il1b reverse	5′-ATC TTT TGG GGT CCG TCA ACT-3′
Il6 forward	5′-CTG CAA GAG ACT TCC ATC CAG TT-3′
Il6 reverse	5′-GAA GTA GGG AAG GCC GTG G-3′
Il10 forward	5′-GCT CTT ACT GAC TGG CAT GAG-3′
Il10 reverse	5′-CGC AGC TCT AGG AGC ATG TG-3′

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

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

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

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

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

RESULTS

NEU activity and NEU1/NEU3 expression in nephritic kidneys of lupus mice. Previously, we showed that *Neu1* message levels and NEU activity increase in the kidneys of nephritic MRL/lpr lupus mice and parallel increases in LacCer and GlcCer (due to catabolism of gangliosides by NEUs) in the kidneys and urine (34). To determine whether increased NEU activity in the kidneys may be a common phenomenon in lupus nephritis (LN), we measured NEU activity in NZM2410 mice at an early disease stage (8–10 wk old) and a mid- to late-disease stage (23–28 wk old). In agreement with results observed in the MRL/lpr lupus strain, NEU activity is significantly increased in the kidneys of mid- to late-disease stage compared with early disease stage (Fig. 1*A*). Of the eight 23–28-wk-old mice, five had 30–500 mg/dl protein, two had trace amounts of protein, and one was negative for protein in their urine. All of the 8–10-wk-old mice were negative for protein or had only trace amounts of protein in their urine. Although some of the 23–28-wk-old NZM2410 mice had low or no measurable protein in their urine, a hallmark of nephritis, we demonstrated previously in the MRL/lpr lupus strain that renal NEU activity increases as disease progresses and is elevated before significant increases in proteinuria and development of nephritis (34). The results in the NZM2410 mice show the same significant increase in renal NEU activity with disease progression. Together, with our results in LN patients (34), these results further support a potential role of NEU-mediated glycosphingolipid catabolism in LN.

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

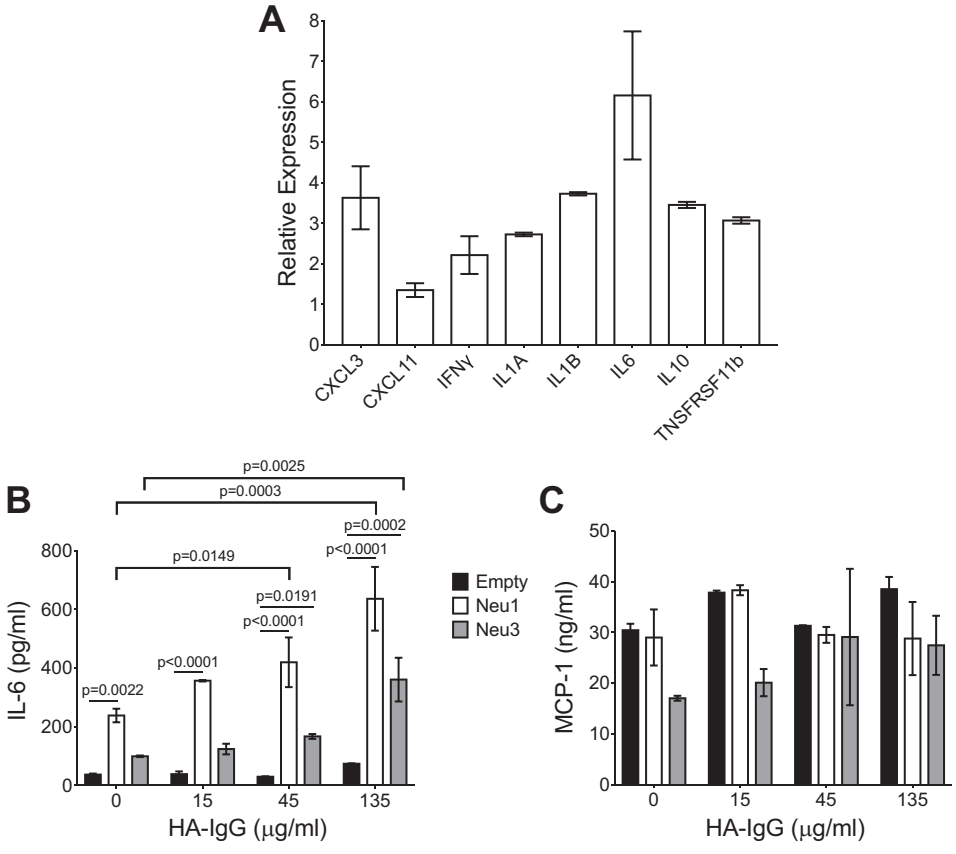

Fig. 2. GSL catabolism upregulates *Il-6* gene expression and IL-6 production by MES13 MCs. *A*: MES13 MCs were cultured with LacCer and GlcCer, mRNA isolated, and cytokine gene expression was measured by real-time PCR in reverse-transcribed mRNA. Data are an average of two or three independent experiments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

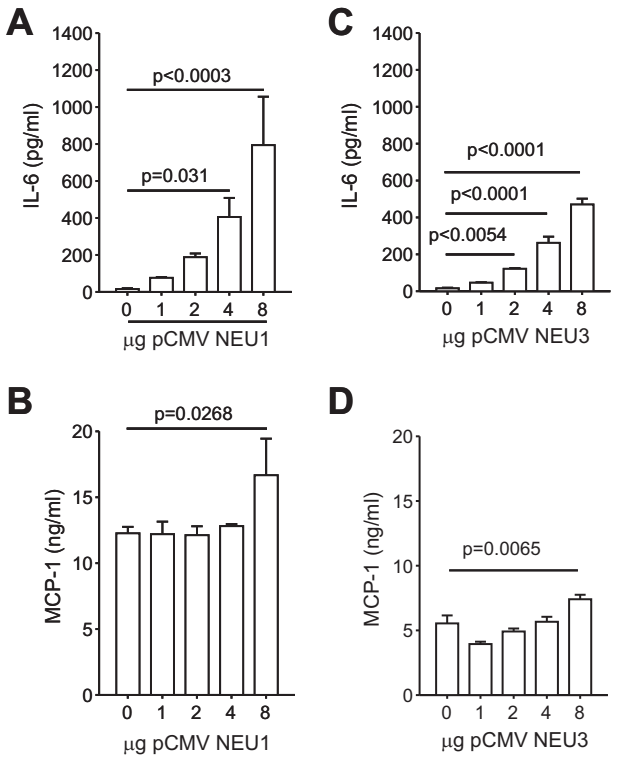

Fig. 3. MES13 MCs produce IL-6 in a dose-dependent manner in response to NEU1 or NEU3 overexpression in the absence of stimulation. MES13 mouse MCs were transfected with increasing concentrations of a NEU1 (*A* and *B*) or NEU3 (*C* and *D*) expression plasmid and media collected 24 h (not shown) and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

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

GSL catabolic pathway stimulated MC IL-6 production. An increase in GSL catabolism results in increased LacCer and GlcCer levels, which are elevated in LN (34). To determine the effects of increased LacCer and GlcCer on MCs, we treated the MES13 mouse MC line with short-chain cell-permeable GSL analogs (C8) of GlcCer and LacCer. After treating MES13 mouse MCs with equal amounts of C_8-GluCer and C_8-LacCer, the expression levels of proinflammatory cytokines and chemokines were determined using a pathway-focused RT^2 profiler PCR Array (SA Biosciences). Approximately 43 proinflammatory cytokines and chemokines were upregulated (twofold or greater compared with vehicle-treated) in the GSL-treated cells (Table 2). Twelve of 43 genes were identified as kidney-relevant proinflammatory factors using Ingenuity Pathways Analysis software. Six of those genes were verified to be consistently increased twofold or greater (Fig. 2*A*) using independently designed and optimized primers (Table 1). These results suggest that elevated GSL catabolism in MCs is sufficient to induce production of inflammatory cytokines that play a major role in renal infiltration of immune cells.

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

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

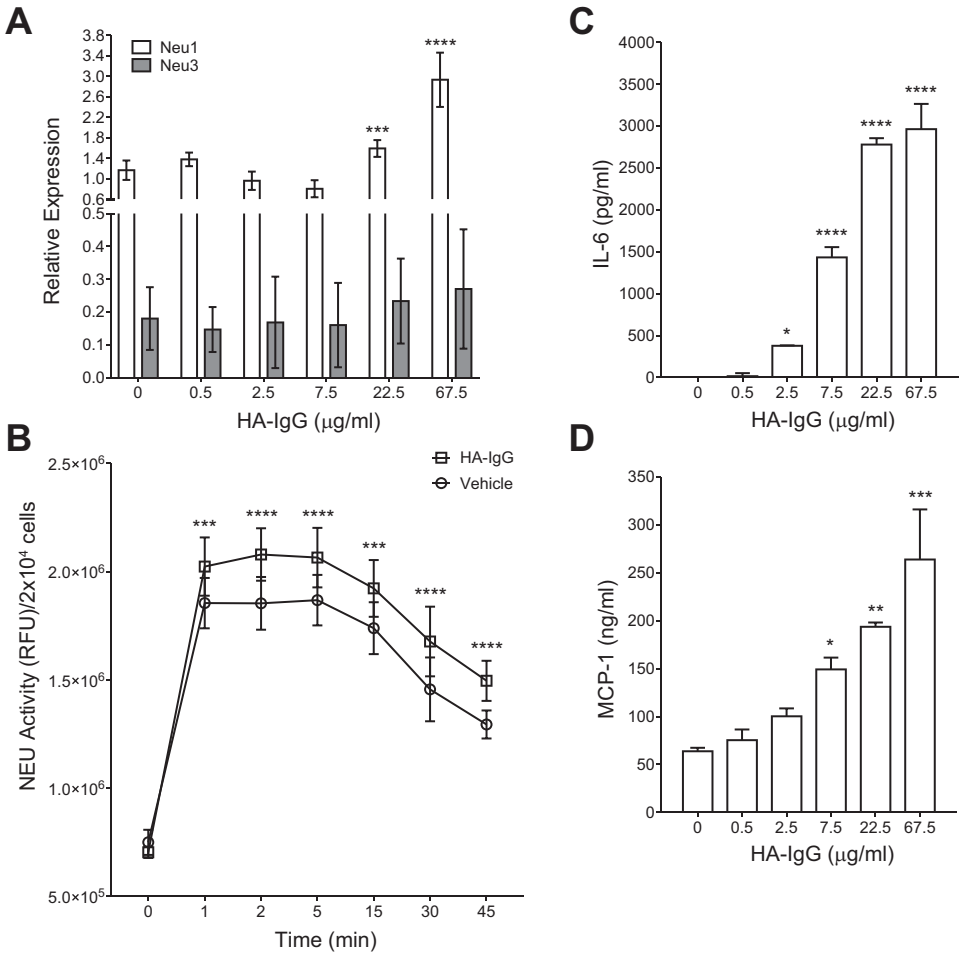

Fig. 4. HA-IgG stimulation of primary MRL/lpr lupus-prone MCs increases *Neu1* message levels, NEU activity, IL-6, and MCP-1 production. Primary MRL/lpr MCs were grown out of glomeruli isolated from prenephritic mice and stimulated with HA-IgG. *A*: cells were treated with increasing concentrations of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

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

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

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

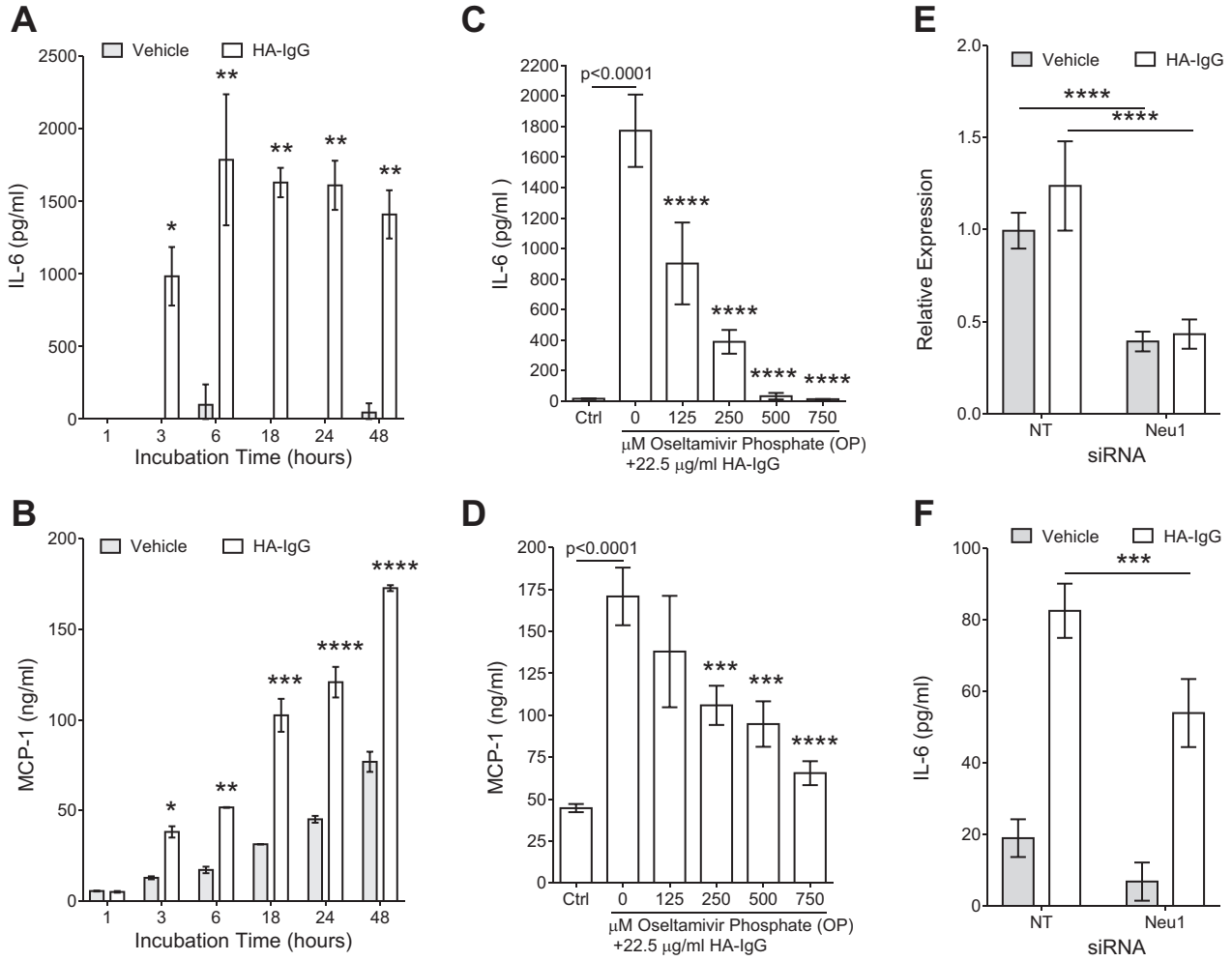

Fig. 5. Inhibiting NEU activity blocks HA-IgG-stimulated IL-6 and MCP-1 production by MRL/lpr lupus prone MCs. *A* and *B*: primary MRL/lpr MCs were treated with 22.5 μg/ml of HA-IgG over time. IL-6 (*A*) and MCP-1 (*B*) levels were measured in media by ELISA and normalized to total protein. *C* and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

activity in IL-6 and MCP-1 production in the primary MCs when stimulated with serum from 18-wk-old nephritic MRL/lpr mice, which contains high levels of IgG (53). IL-6 production by the MCs increased as the percentage of MRL/lpr serum increased, which was significant with 10% lupus serum (Fig. 6*A*). Serum from C57BL6 (B6) healthy mice had no effect. Neither MRL/lpr serum (5 to 20%) nor B6 serum (20%) resulted in significant production of MCP-1 (Fig. 6*B*). Importantly, as with HA-IgG stimulation, OP significantly suppressed lupus serum-stimulated IL-6 production in a dose-dependent manner 3 h (Fig. 6*C*) and 6 h (data not shown) after stimulation. These results indicate that IL-6 production of lupus prone MCs following activation by lupus serum is mediated by NEU activity. Combined with the results above with HA-IgG, it is likely that NEU may specifically mediate IL-6 production by lupus-prone MCs activated by circulating IgG-containing immune complexes.

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

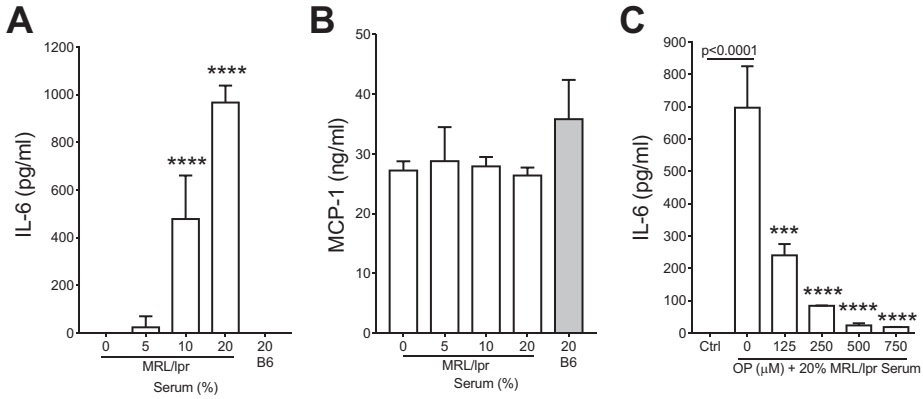

Fig. 6. Inhibiting NEU activity blocks lupus serum-stimulated IL-6 production by MRL/lpr lupus prone MCs. Primary MRL/lpr MCs were treated with increasing concentrations of MRL/lpr serum or B6 serum for 24 h. IL-6 (*A*) and MCP-1 (*B*) levels in media were measured by ELISA and normalized to total protein.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

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

DISCUSSION

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

MCs occupy the central stalk of the glomerulus and interact with endothelial cells and podocytes. It gives structural support and contributes to the regulation of glomerular filtration, occupying ~40% of the total cells in the glomerulus. MCs exhibit both innate and adaptive immune properties, such as cytokine production and phagocytosis and are important early drivers in promoting renal inflammation in LN. Their response to pathological insult is associated with key events in glomerular dysfunction. Activating MCs by various methods leads to production of a number of proinflammatory cytokines and chemokines, including IL-6 and MCP-1. Here, we demonstrated that increasing LacCer and GlcCer levels in the MES13 MC line upregulates a number of cytokine genes, including *Il-6*. IL-6 plays an important role in the progression of lupus nephritis. Lupus mice treated with anti-IL-6 antibodies or with a homozygous knockout of IL-6 had significantly improved survival and reduced/delayed nephritis that included reduced inflammatory cell infiltration (9, 23). Of translational significance, LN patients with active disease were shown to have increased renal and urinary IL-6 levels, with both MCs and inflammatory cells contributing to elevated renal levels (13, 16, 18, 36, 46, 48). The elevated renal IL-6 levels are thought to be largely due to infiltrating inflammatory cells in LN patients. However, on the basis of studies of IL-6 in lupus mouse strains (9, 19, 22), IL-6 produced by MCs appears to play a role in promoting the production of other cytokines in the kidney to attract inflammatory cells following immune complex deposition early in disease, which is difficult to study in human patients.

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

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

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

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

*Kidney-relevant upregulated cytokines/chemokines.

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

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

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

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

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

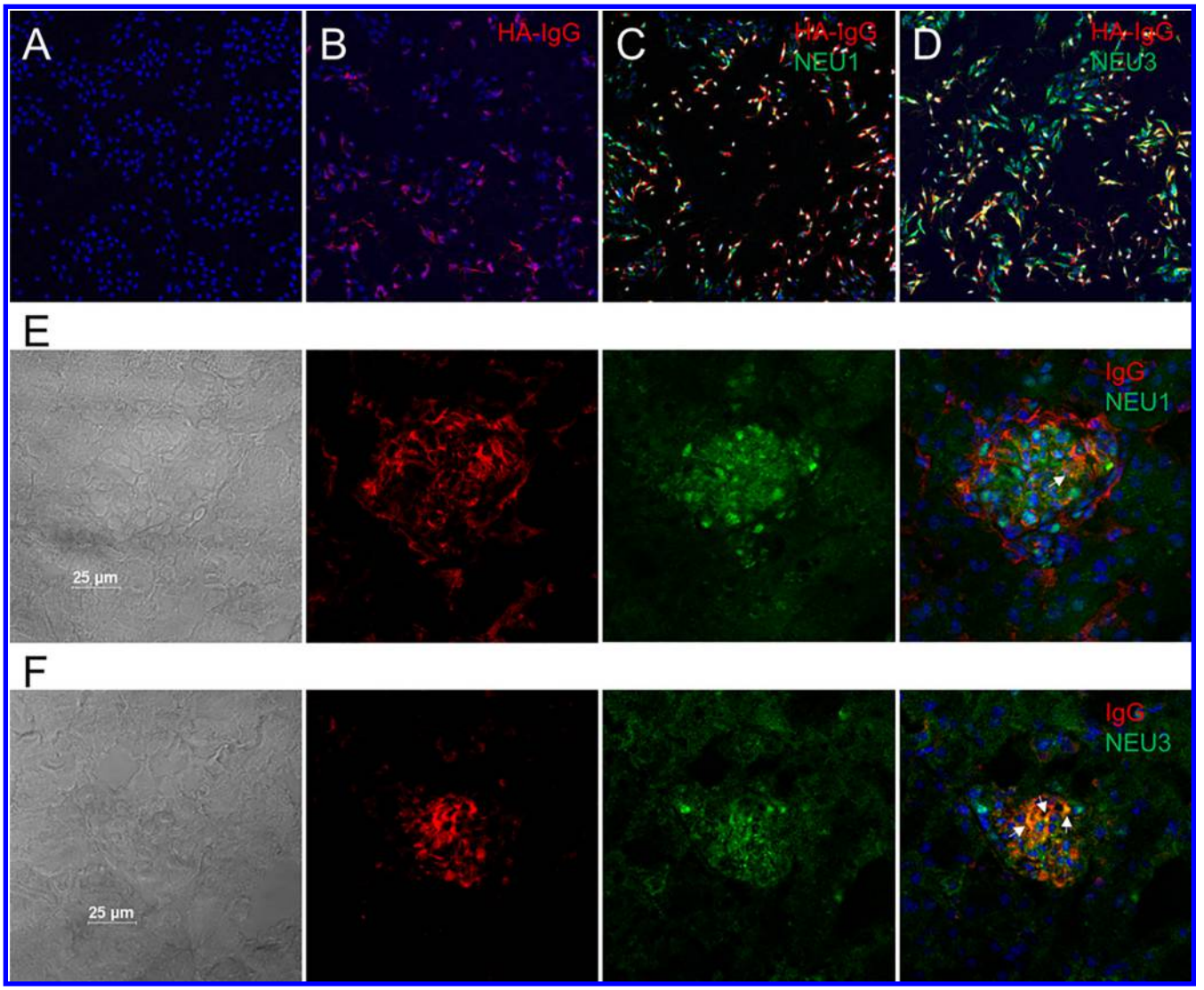

Fig. 7. NEU1 and NEU3 expression overlaps with IgG deposits on the surface of MRL/lpr lupus-prone MCs and in the glomeruli of nephritic MRL/lpr mice. MRL/lpr lupus-prone MCs were fixed (but not permeablized) and stained with isotype (*A*) or HA-IgG for 1 h followed by Alexa Fluor 594 goat anti-mouse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

response to immune complex deposition in LN (5). MCs express FcRs that bind IgG, and HA-IgG was shown to colocalize with Fcγ receptors (FcγR) III or IV on MCs and trigger production of inflammatory mediators, including IL-6 and MCP-1 (37, 38, 45). However, studies with FcγR-deficient MRL/lpr mice suggest that development of nephritis in this strain is independent of FcγR (27). Immune complexes also can signal through Toll-like receptors (TLRs), which are expressed by MCs and have been implicated in the development of LN (12, 21, 24, 35, 44). NEU1 was shown to colocalize with TLR2 and TLR4 on the surface of macrophages and dendritic cells and mediate signaling (1, 4, 10). TLRs 1–4 are expressed by cultured MRL/lpr MCs (35). Alternatively, NEU activity may mediate IgG activation of MCs through an unknown receptor complex or through IgG receptor-independent effects. Our NEU1 and NEU3 overexpression results in the MES13 MCs suggest that NEU activity can activate an IL-6 signaling pathway in the absence of exogenous stimulant.

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

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

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

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

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