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TITLE: Glycosphingolipids as Therapeutic Targets and Biomarkers of Lupus Nephritis

PRINCIPAL INVESTIGATOR: Tamara Nowling, PhD

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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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14. ABSTRACT

All urine and serum samples for the full biomarker study in lupus nephritis patients that have responded and failed therapy have been identified and collected. Prior to beginning the full study, we tested/streamlined the workflow and identified putative lipid/protein candidates in a pilot study among the full study patients that had flare and non-flare urine samples. We also collected all the renal biopsies and are currently performing the MALDI/FTICR analyses for correlating renal lipid expression with clinical disease measures. We completed the backcrossing of the Neu1 knockout from the C57BL/6 mice to the lupus mouse strain B6.SLE1/2/3 and are breeding to obtain sufficient numbers of mice for the genetic study analyzing the effects of reducing NEU1 levels on disease development. To identify mechanisms by which NEU-mediated GSL catabolism impacts renal function and dysfunction in lupus, additional studies were performed on mesangial cells from mice in our proposed studies. Results include demonstrating that 1) that NEU activity mediates IL-6 production in mesangial cells, 2) NEU1/3 expression co-localizes with renal IgG deposition, and 3) mesangial cells from Neu1 knockout mice have reduced NEU activity and produce significantly reduced levels of IL-6 compared to Neu wild-type.

15. SUBJECT TERMS
Lupus nephritis, Glycosphingolipid, Biomarkers, Neuraminidase

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
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TABLE OF CONTENTS

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

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

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

Lupus nephritis, Glycosphingolipid, Biomarkers, Neuraminidase

3. **ACCOMPLISHMENTS:** The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants 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. Local IACUC and ACURO approvals have been obtained for these studies (subtask 1, completed). Due to limited availability of the MRL/lpr mice from vendors, we have had to purchase mating pairs and breed this strain in our facility to generate the number of mice needed for this treatment study. Therefore, subtask 2 (treatment of MRL/lpr mice) and subtask 3 (treatment of B6.SLE1/2/3 mice) have been switched in the timeline on the SOW. B6.SLE1/2/3 mice will begin treatment in the next few months. We anticipate having all samples collected from the B6.SLE1/2/3 treated mice within the time frame indicated in the SOW for subtask 2. The MRL/lpr treatment study will begin once the B6.SLE1/2/3 samples have been collected and are expected to be completed within the timeframe indicated for subtask 3 in the SOW.

Major Task 2: Genetic knockout of Neu1 in lupus mice. Local IACUC and ACURO approvals have been obtained for this study (subtask 1, completed). The B6 Neu1 knockout has been backcrossed onto the B6.SLE1/2/3 strain (subtask 2, completed). Breeding of B6.SLE1/2/3 $Neu1^{+/-}$ mice is ongoing for generating the B6.SLE1/2/3 $Neu1^{+/+}$, $Neu1^{+/-}$ and $Neu1^{-/-}$ mice needed for the remaining subtasks. This Major task is ahead of schedule indicated in the SOW timeline.

Major Task 3: GSLs as a predictor of response therapy. Local IRB and HRPO approvals have been obtained (subtask 1, completed). Patients that meet inclusion criteria have been identified and the urine samples have been obtained from the biorepository (subtask 2, completed). Fifteen urine samples were used to isolate and analyze exosomes by MALDI-FTICR (subtask 3), proteomics (subtask 5), and western blot (subtask 6) as a pilot test of the work flow and analyses. Exosome isolation and subtask analyses are proceeding with all urine samples and is on-schedule as outlined

in the SOW timeline.
Major Task 4: Association of renal and urine GSLs with renal function. Patients with available biopsy samples have been identified and samples obtained (subtask 1, completed). Biopsies are currently being sectioned for MALDI-FTICR analysis (subtask 2, in progress). Major task 4 is on-schedule as indicated in the SOW timeline.

What was accomplished under these goals?

The major activities completed for the first year of the grant included: 1) obtaining IACUC and IRB approvals; 2) obtaining the B6 Neu1 knock out strain from a collaborator and back crossing the Neu1 knockout onto the B6.SLE1/2/3 lupus strain; 3) breeding mouse strains B6.SLE1/2/3 (wild type and $Neu1^{+/-}$) and MRL/lpr (ongoing) to generate mice needed for the treatment and genetic studies; 4) identification of patients and obtaining samples needed for all urine and renal biopsy analyses; 5) exosome isolation and analysis of urine samples from pilot group of patients to test work flow; and 6) sectioning of renal biopsies for the MALDI-FTICR analyses. The specific objectives for this past year were to obtain all the necessary materials (including generating the B6.SLE1/2/3 Neu1 knockout) so that we are poised for proceeding with all mouse and human patient sample analyses, test the work flow for the exosome isolation and downstream analyses, and to begin the oseltamivir treatment study for one of the mouse strains. Most tasks are either on-schedule or ahead of schedule as proposed in the SOW.

The pilot study analyzed exosomes isolated from flare and non-flare urine samples from five lupus nephritis (LN) patients and samples from five lupus patients without nephritis. The exosomes were divided for use in proteomic, MALDI (lipid), and western immunoblot analyses. Proteomics results from the analyses on the pilot urine samples identified ten proteins that were detected at greater levels in urine exosomes from the flare samples of the five LN patients compared to their non-flare samples and compared to samples from five lupus patients without nephritis. The levels of the five most abundant of those ten proteins are shown in Fig. 1.

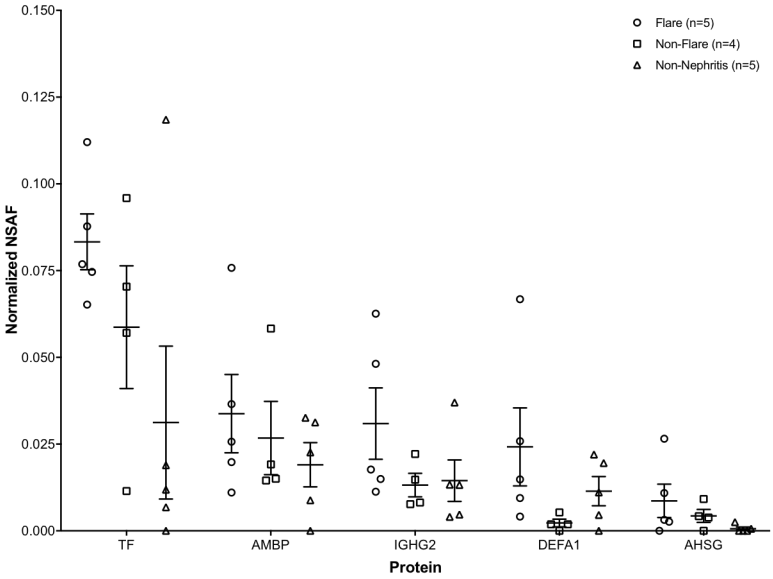


Fig. 1. Proteins that are more abundant in urine exosomes from LN patients during a flare compared to during a non-flare period and compared to lupus patients without nephritis. Equivalent amounts of exosomes based on protein concentration were digested with trypsin, processed and separated on a 2D+NanoLC Ultra system. Protein was analyzed for relative abundance and identified using ScaffoldQ+(v4.6) and mascot(v4) software. NSAF (normalized spectral abundance factor)

In addition, there were four proteins that were detected at higher levels (Fig. 2) and six proteins detected at lower levels (Fig. 3) in the LN patients (both flare and non-flare samples) compared to lupus patients without nephritis. These results identify potential flare-specific proteins and nephritis-specific proteins that are over-represented in LN patient urine exosomes as well as proteins that are under-represented in LN patient urine exosomes compared to non-nephritis lupus patients. This pilot study provides putative proteins that can serve as a primary focus in the full urine exosome study as well as identifying additional proteins specific to therapeutic response/flare.

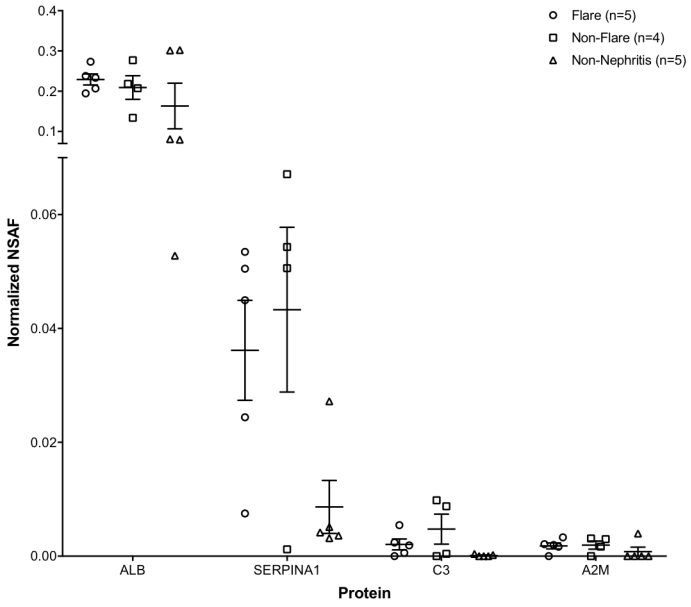


Fig. 2. Proteins that are more highly represented in urine exosomes from LN patients during flare and non-flare periods compared to lupus patients without nephritis. Proteins were identified as described in Fig. 1 legend. NSAF (normalized spectral abundance factor)

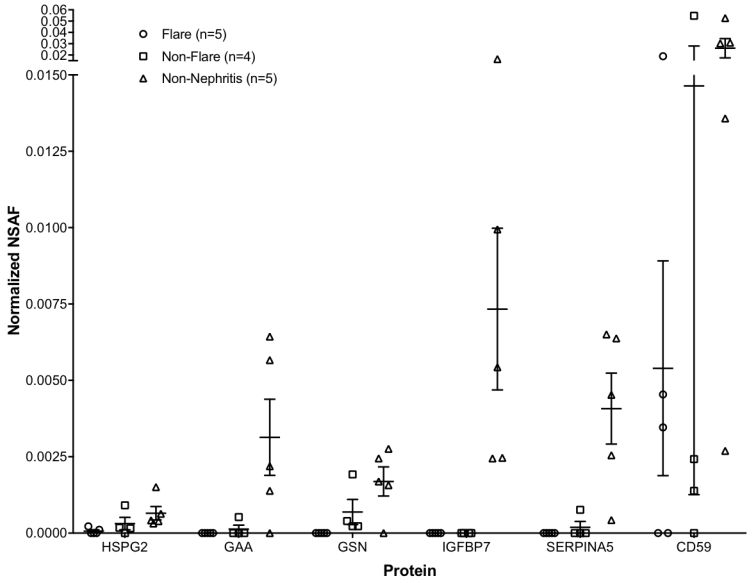
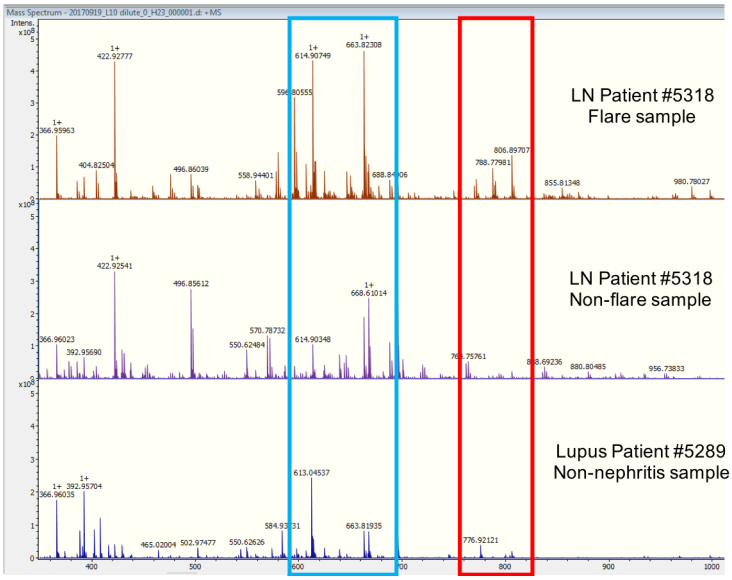


Fig. 3. Proteins that are less abundant in urine exosomes from LN patients during flare and non-flare periods compared to lupus patients without nephritis. Proteins were identified as described in Fig. 1 legend. NSAF (normalized spectral abundance factor)

Results of the MALDI-FTICR mass spectrometry analyses of these samples showed clear differences in a variety of lipids in the samples taken during a flare compared to samples taken during quiescent disease (non-flare) in the lupus nephritis patients. There was a greater abundance of lipids in general as well as increased levels of specific lipids in the flare compared to the non-flare samples. The samples from the lupus patients without nephritis tended to have lower overall abundance of lipids compared to both the flare and non-flare samples from the LN patients. The lipid mass spectrum results from a representative LN patient (flare and non-flare) and a lupus patient without nephritis are shown in Figure 4. Fig. 4A shows lipids measured between 350 and 1000 mass to charge ratio (m/z). The m/z ratios of 550-700 (blue box in Fig. 4A) and 750-820 (red box in Fig. 4A) are expanded in Fig. 4B and 4C, respectively, to show differences in specific lipids (peaks) between the samples. Identification of specific lipids represented by the m/z peaks is on-going.

A



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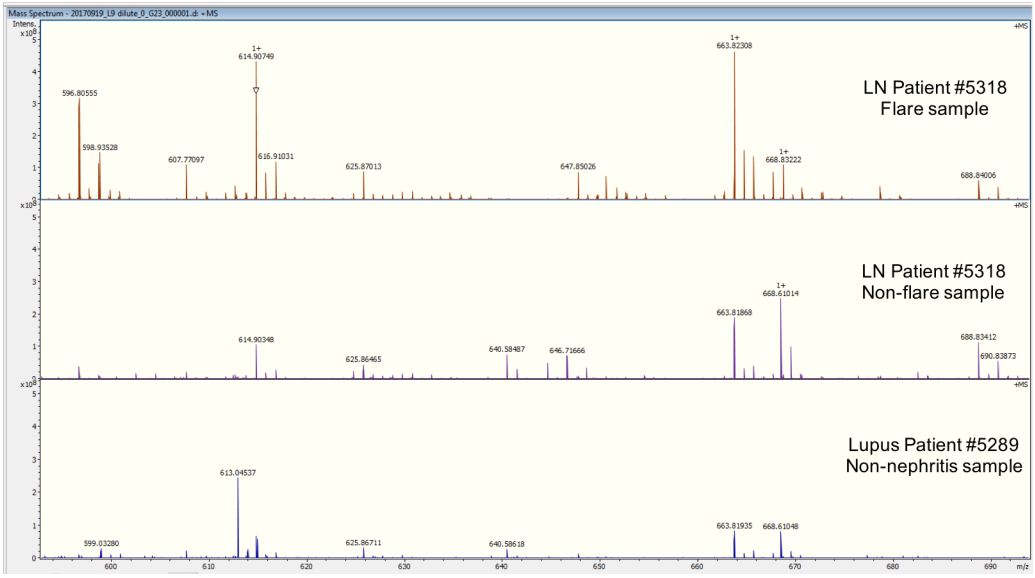

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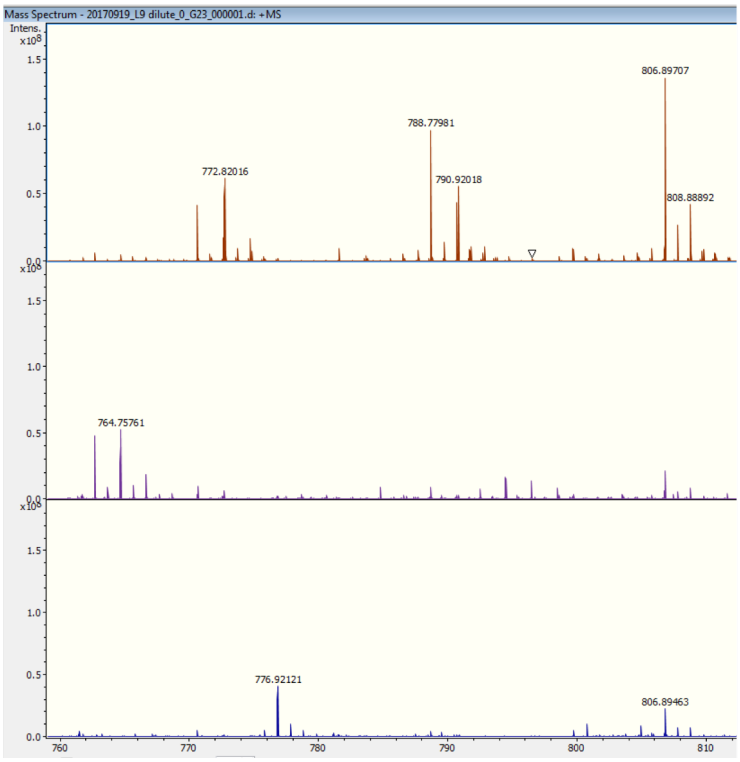

LN Patient #5318
Flare sample

LN Patient #5318
Non-flare sample

Lupus Patient #5289
Non-nephritis sample

Figure 4. Representative mass spectrum of flare and non-flare samples from LN patient #5318 and from non-nephritis lupus patient #5289. Equivalent amounts of exosomes based on protein concentration were used for lipid extraction. Lipids were spotted on a steel plate, mixed with 2,5 dihydroxybenzoic acid (DHB) matrix and measured by MALDI-FTICR mass spectrometry. The m/z charge ratios spanning 350-1000 in (A) is zoomed in for m/z spanning 550-700 in (B) and 750-820 in (C) to better visualize/identify individual peaks. X-axes, m/z; Y-axes, relative abundance.

A western immunoblot was used to demonstrate the presence of exosome-specific protein Alix. Exosomes were pooled from individuals within each group from a total of 10 ml urine and total protein extracted from exosomes were analyzed on the immunoblot. Alix was more abundant in the lupus nephritis flare samples, which is in agreement with previous observations that urine from nephritic individuals contain more exosomes. Our results also indicate that more exosomes are present in samples from lupus nephritis patients during a flare. We now have access to an exosome analyzer at our institution with the capability of quantifying exosome numbers and will use this equipment to confirm immunoblot observations. We did not detect NEU1 in the exosome samples in any of the groups, which may be below the level of detection by immunoblot. However, NEU1 was detected in the proteomics analysis in a few patients. Therefore, in addition to the proteomics discovery screen to identify proteins, we will perform a proteomics analysis to specifically measure NEU proteins and ignore the background of all the other proteins that are typically analyzed as part of the discovery screen (as represented in Figs. 1-3).

In performing the pilot study, we refined the workflow and determined that albumin and associated hemoglobin (and heme lipids) are highly abundant, especially in the LN flare samples. Therefore, we will incorporate an extra simple purification step using a size exclusion spin column that will remove non-exosome associated albumin and red blood cells. This will avoid any masking of less

abundant proteins and lipids that are significantly different among the samples. In addition, our institution recently acquired an exosome analyzer, which we are incorporating into our workflow to assess size and more precisely quantify exosome numbers. This will allow for a more accurate normalization of measures/quantifications in each of the subtask analyses. We are proceeding with exosome isolation and downstream analyses of urine samples of the full study to identify biomarkers for therapeutic response.

Other achievements that have stemmed from the DoD supported studies (but not directly part of the proposal) include mechanistic studies that have evolved from working with the mice being generated and studied as part of the DoD proposal. Glomeruli were isolated from the Neu1 knockout mice and MRL/lpr lupus mice and primary mesangial cells grown out to establish primary cell lines. These primary mesangial cells were used for *in vitro* oseltamivir treatment studies and other mechanistic studies. The major findings are that NEU activity mediates IL-6 production by mesangial cells stimulated with an immune complex mimic (HA-IgG) or lupus serum and that production is blocked by the NEU activity inhibitor oseltamivir phosphate (which we are using for the DoD funded treatment study in the lupus strains). Some of these results have been included in a manuscript that is expected to be accepted to the American Journal of Physiology: Renal Physiology upon requested minor revisions following the initial review. A draft of the submitted revised manuscript is included in the appendix. Studies with the Neu1 knockout mesangial cells show that cells heterozygote for Neu1 have significantly reduced NEU activity (Fig. 5A) and significantly reduced IL-6 production (Fig. 5B) upon stimulation with HA-IgG, lupus serum, and/or LPS. These results will lend additional mechanistic insight to results obtained through the genetic study of the B6.SLE1/2/3 Neu1 knockout mice and the oseltamivir treatment study in the lupus mouse strains funded in the DoD proposal.

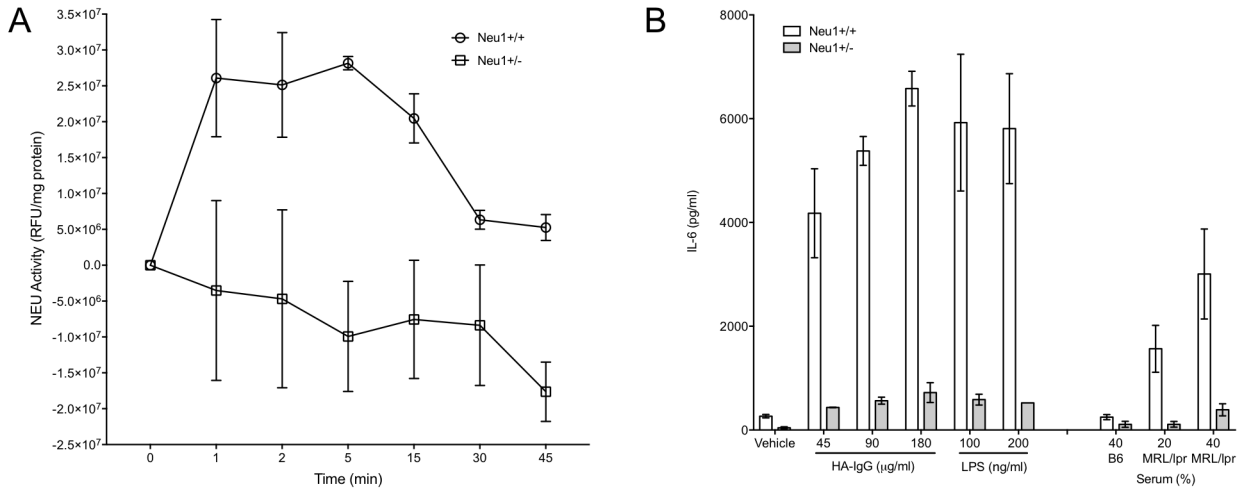

Figure 5. Mesangial Cells heterozygous for Neu1 have reduced NEU activity and IL-6 production. A) C57BL/6 Neu1+/+ and Neu1+/- mesangial cells were stimulated in the presence of vehicle (control), heat aggregated IgG (HA-IgG, mimic of immune complex deposition), LPS; or serum from a wild-type C57BL/6 mouse (control) or a late disease stage MRL/lpr lupus mouse, as indicated in the graphs. IL-6 was measured in the media. B) NEU activity was measured on live cells after stimulation with 45 μg/ml HA-IgG. Measures were performed 24 h after stimulation for both experiments.

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 College of Rheumatology conference in Washington, D.C. and the Southeastern Regional Lipid Conference in Cashiers, N.C. in November, 2016 and presented research findings on the *in vitro* mechanistic studies. These studies were supported in part by this grant as described above. In addition, the PI met with collaborators at the two conferences and met with the co-investigators 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 College of Rheumatology conference in Washington, D.C. and the Southeastern Regional Lipid Conference in Cashiers, N.C. in November, 2016 on research findings of *in vitro* oseltamivir treatment of primary mesangial cells, which are supported in part as an extension of the studies proposed in this grant.

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

For Major task 1, we plan to complete the treatment study and collection of samples (subtask 3) and a majority of the analyses of the B6.SLE1/2/3 mice (subtask 4). We also plan to begin the treatment study and collection of samples of the MRL/lpr mice (stubtask 2).
For Major task 2, we plan to complete the collection of samples and all analyses of the B6.SLE1/2/3 Neu1 knock out genetic study (subtask 3 and 4).
For Major task 3, we plan to complete the isolation of urine exosomes from all patient samples and complete the MALDI-FTICR analyses on the pre-treatment samples and begin MALDI-FTICR analyses on a majority of the post-treatment samples (subtask 3), the Lipidomics measurements (subtask 4) will begin towards the end of the next year after MALDI analyses of the samples are completed. We will also complete the proteomics analyses for the pre-treatment samples and a majority of the post-treatment samples (subtask 5), and complete the western (and exosome analyzer) analyses of all samples (subtask 6).
For Major task 4, we plan to complete the sectioning of all biopsy samples and the MALDI-FTICR analyses (subtask 2) and begin data analyses (subtask 3).

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?

We have generated a global knock out of the neuraminidase 1 (Neu1) gene on the background of the lupus prone strain B6.SLE1/2/3. These mice have reduced levels of the NEU1 protein in tissues where this gene is expressed. Although we are primarily interested in the effects of reducing NEU1 protein levels in the kidney, this mouse strain will benefit lupus researchers interested in studying the effects of reducing NEU1 in other tissues or cell types, such as T cells where NEU1 also has been shown to play a role in the progression of lupus.

What was the impact on other disciplines?

Nothing to Report

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

We encountered a delay in obtaining MRL/lpr mice due to availability of the number and gender of the mice required for our treatment studies and re-budgeting (see below). Jackson Laboratories from which we obtain the mice require a substantial lead time to breed the large number of mice needed as they do not keep a large number of mice from this strain on hand. In addition, they limit the number of mice from this strain per order. Therefore, we will be breeding this strain to generate the mice needed for the oseltamivir treatment study. Due to the delay, we switched subtask 2 (treatment of MRL/lpr mice) and subtask 3 (treatment of B6.SLE1/2/3 mice).

Changes that had a significant impact on expenditures

Nothing to Report

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

Significant changes in use or care of human subjects

Nothing to Report

Significant changes in use or care of vertebrate animals.

Nothing to Report

Significant changes in use of biohazards and/or select agents

Nothing to Report

6. **PRODUCTS:** 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**. 2017. Neuraminidase Activity mediates IL-6 production by activated lupus prone mesangial cells. Journal of Physiology: Renal Physiology, *accepted pending revisions*.
(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, Jessalyn Rodgers, Leah Siskind, Richard Drake. 2016. NEU1-mediated glycosphingolipid catabolism regulates the function of mesangial cells. Southeastern Regional Lipid Conference, Cashiers, NC.

Tamara Nowling, Kamala Sundararaj and Leah Siskind. 2016. Immune Complex-Induced IL-6 Production by Lupus Prone Mesangial Cells is Mediated by Neuraminidase Activity. American College of Rheumatology Annual Meeting, Washington, D.C.

- **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**

A Neu1 knockout on the lupus prone mouse strain B6.SLE1/2/3 was generated during the first year of the award. This can serve as a model for several areas of investigation in the lupus field.

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 wrote all IACUC and IRB protocols and obtained required approvals, directed planning of all experiments and analyses, met with and coordinated patient pilot urine exosome sample analyses with co-investigators, and assisted with and/or provided guidance with backcrossing Neu1 knockout onto the B6.SLE1/2/3 strain, exosome western analyses.

Name: Jessalyn Rodgers, MS
Project Role: Research Specialist
Nearest person month worked: 12
Contribution to Project: Ms. Rodgers' main duties have involved performing the backcrossing of the Neu1 knockout onto the B6.SLE1/2/3 strain (breeding and genotyping of mice), exosome western analyses, and sectioning of renal samples. 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: Post-doctoral scholar
Nearest person month worked: 12
Contribution to Project: Dr. Sundararaj aliquoted patient urine samples and inventoried all patient samples, assisted with exosome western analyses and mouse genotyping, and has performed the majority of the mechanistic studies- isolating glomeruli and establishing primary mesangial cell lines from wild type and Neu1 knockout mice, culturing and performing all in vitro oseltamivir treatment studies and analyses.

Name: Rick Drake, PhD
Project Role: Co-investigator
Nearest person month worked: 1.2
Contribution to Project: Dr. Drake performed the MALDI-FTICR analyses on the set of pilot exosome samples and the renal biopsies.

Name: Mike Janech, PhD
Project Role: Co-investigator
Nearest person month worked: 1.2
Contribution to Project: Dr. Janech performed the proteomic analyses on the pilot exosome samples. He has also helped in refining the purification of the exosome samples.

Name: Jim Oates, MD
Project Role: Co-investigator
Nearest person month worked: 1.05
Contribution to Project: Dr. Oates assisted with preparing the IRB protocol and identified patients meeting the inclusion criteria that had samples available for the proposed studies.

Name: Beth Wolf, PhD
Project Role: Co-investigator
Nearest person month worked: 1.2
Contribution to Project: Dr. Wolf assisted with analyzing the mesangial cell data and the preliminary data obtained from the pilot exosome MALDI-FTICR and proteomic analyses.

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: In the past year, Dr. Nowling had effort end on two projects: MUSC Foundation Pilot Grant and ADVANCE-PAID.

James C. Oates, MD: In the past year, Dr. Oates had effort end on 2 projects: Takeda MLN9708_101 and BMS Scleroderma. *He is now providing effort on two new studies (no overlap with DoD studies):*

1) MUSC Foundation for Research and Development (3/16/17-3/15/18), Exploring Complement Activation in Patients with Lupus Nephritis (In vitro Complement Deposition Assay. The goal of this project is to determine differential complement activation in patients with inactive versus active lupus nephritis.

Role on project: PI, 0 CM

2) NIH/NIAMS P60 AR062755-01 (9/25/17-8/31/22), MCRC for Rheumatic Diseases in African Americans. The goal of the MCRC is the advancement of knowledge with respect to African Americans who have, or who are at risk of developing systemic lupus erythematosus, systemic sclerosis, and other debilitating rheumatic diseases. The purpose of the Patient Resource Core is to recruit and retain a well-characterized group of SLE or scleroderma patients and healthy controls for clinical and biological studies is the associated projects and future projects. Project 2 will test the hypothesis that there are gene-environment interactions leading to the development of SLE and that comparing Gullah versus Sierra Leoneans will allow identification of key pathogenic factors in SLE.

Role on project: PI of Patient Core and Project 2, 1.37 CM

Bethany Wolf, PhD: In the past year, Dr. Wolf has had effort end on two projects: P60AR062755, R01NS071867. *She is now providing effort on three new studies (no overlap with DoD studies):*

1) NIH/NIAMS P30AR072582-01 (9/15/17-8/15/22), Improving Minority Health in Rheumatic Disease, Methodology Core. The goal of this CCCR is the advancement of knowledge with respect to African Americans who have, or who are at risk of developing, systemic lupus erythematosus, systemic sclerosis, and other debilitating rheumatic diseases. The overall objective of the Methodology Core is to provide rigorous methodological and biostatistical support to the MCRC investigators and to lead investigations into racial/ethnic components of rheumatic disease that are focused in methodological areas including studies of gene x gene and gene x environment interactions.

Role on Project: Director of Methodology Core, 2.4 CM

2) NIH K23HL135263-01 (7/15/17-4/30/20), The Role of Circulating Micro RNAs in ARDS Pathogenesis and Outcomes. The goals of the proposed study are two-fold: (1) evaluate the association between plasma levels of miR-887 and miR-15a and their secreted with sepsis-related ARDS and secondary outcomes of ARDS such as mortality and (2) to determine the impact of sepsis-related stimuli on expression of miR-887 and miR-15a in human microvascular endothelial cells (HMVECs).

Role on Project: Co-I, 0.36 CM

3) NIH R01HL133751 (8/15/17-6/30/21), Role of pericytes in scleroderma skin and lung fibrosis. We hypothesize that activated pericytes contribute to myofibroblast populations in both skin and lungs, and pericytes are an important target cell for the anti-fibrotic activities of E4. To investigate these hypotheses, we propose to (1) compare the role of lung and skin pericytes as myofibroblast progenitors in murine models of fibrosis, (2) compare the distribution and function of pericytes from normal and fibrotic lung and skin from scleroderma patients, and (3) determine if skin and lung pericytes are a target of the novel anti-fibrotic peptide E4.

Role on Project: Co-I, 0.6 CM

Michael Janech, PhD: In the past year, Dr. Janech has had effort end on one project: N00014140361.

Richard Drake, PhD: In the past year, Dr. Drake had effort end on three projects: R41 GM112750-01A1, R21 CA175894-01A1, and R21 CA160192-01. *He is now providing effort on two new studies (No overlap with DoD studies):*

1) *NIH/NIMHD U54 MD010706-02 (7/8/16-3/31/21), Medical University of South Carolina Transdisciplinary Collaborative Center in Precision Medicine and Minority Men's Health. The goal is to understand the impact of social determinants and stress on tumor microenvironment. and the association with prostate cancer progression in African American and Caucasian men.*
   Role on Project: Co-I, 1.2 CM
2) *NIH/NCI P01 CA203628-02 (5/1/16-4/30/21), Development of Novel Cancer Therapeutics by Targeting Sphingolipid Signaling. The lipidomic analysis core will support the different projects for quantitative sphingolipid analysis and MALDI tissue imaging analysis of sphingolipids.*
   Role on Project: Co-I, 1.2 CM

What other organizations were involved as partners?

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

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

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

9. APPENDICES:

Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.

- Submitted manuscript: "Neuraminidase Activity mediates IL-6 production by activated lupus prone mesangial cells". Kamala Sundararaj, Jessalyn Rodgers, Marimuthu Subathra, Leah J. Siskind, Evelyn Bruner and Tamara K. Nowling

“Neuraminidase Activity mediates IL-6 production by activated lupus prone mesangial cells”

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Running head: Neuraminidase activity in lupus mesangial cells

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Abstract

The development of nephritis is a leading cause of morbidity and mortality in lupus patients. While the general pathophysiological progression of lupus nephritis is known, the molecular mediators and mechanisms are incompletely understood. Previously, we demonstrated that the glycosphingolipid 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 that 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.

Keywords

Lupus nephritis

Mesangial cells

Neuraminidase

IL-6

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 (32, 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 to 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), NEUs result in upregulation of lactosylceramide that produces cytokines and chemokines, which then causes 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 approximately 40-fold higher than *Neu3* and 80-fold higher than Neu4 (52).

Here, we provide additional evidence that renal NEU activity is elevated and NEU1 and NEU3 is 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 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. We further 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 pathway. 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 weeks 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. Gender of mice used to generate primary MRL/lpr mesangial cell lines is described below. Mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and were maintained on a 12-hour (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).

Generation and culturing of primary mesangial cell lines

Kidneys were removed from three 6-week-old (pre-nephritic) 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 carefully 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% fetal bovine serum, 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 approximately 4 weeks with medium changed every 4-5 days. After three weeks, 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 females and one male mice. The second line was generated from three female mice. Results from experiments stimulating each line and with the NEU inhibitor oseltamivir phosphate showed similar trends with data presented from the female line. Each primary MC line was characterized by flow cytometry at passage five, by IHC at passage six, and by RT-PCR at passages 5-9. Cell lines used were confirmed positive for MC markers (α*SMA* and *PDGFR*β), and negative for endothelial cell markers (*CD144* and *CD31*) and podocyte cell markers (*nephrin* and *synaptopodin*). Lines used for experiments were 93% positive for α*SMA* by flow cytometry. RTPCR results showed an average across passages 5-9 relative expression levels of 16.5 for α*SMA* and 1.5 for *PDGF*β. *CD31* and *CD144* did not show amplification until 32-33 cycles and were 0.04 and 0.06, respectively, relative to α*SMA* and *PDGF*β. *Nephrin* was below the level of detection. All cell lines tested negative for mycoplasma. Cells at passages 6-9 were used in all experiments. MC lines were shown to express *Neu1* and *Neu3* with message levels of *Neu1* being 12-14-fold higher than *Neu3*. For the siRNA studies, cells were transfected with 200 nM siGENOME SMARTpool mouse Neu1 siRNA or non-target siRNA (Dharmacon, St. Louis, MO) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) 16 h prior to stimulation with heat aggregated IgG or vehicle. Twenty-four hours after stimulation media was collected and analyzed for

cytokine production and cells were collected used to measure total protein and Neu1 and Neu3 message levels as described below.

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 polyacrylamide gel electrophoresis. MCs were treated with increasing concentrations of HA-IgG or vehicle (sterile PBS), or with increasing amounts of serum from 16-18-week-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 to 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 Inc., Santa Cruz, CA) was added to the culture medium at the concentrations indicated in the figures for 24 h prior to stimulation with 22.5 μg/ml HA-IgG or 20% MRL/lpr serum for 6 h. Media was 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 is 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.

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 x 10^6 cells were plated in 10 cm^2 dishes in complete growth media. Forty-eight hours after plating, the media was 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 6-well plates one day prior to 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 was 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 measured for normalizing IL-6/MCP-1 levels. Trends were the same at 24 h and 48 h time points; data from 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 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.

Enzyme-Linked Immunosorbent Assay (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 to transfections using lower amounts of the NEU1 and NEU3 expression plasmids. Concentrations of IL-6 and MCP-1 for all experiments were normalized to total cellular protein content to account for any effects of treatments. Final concentrations presented were adjusted to an equivalent of 25 μg of protein after normalization for comparison across experiments.

Semi-quantitative real-time RTPCR assays

RNA was isolated from MCs using the RNeasy kit (Qiagen, Hilden, Germany) following manufacturer's directions and cDNA was reverse transcribed with 0.5–1 μg RNA using the iScript cDNA Synthesis kit (BioRad, 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, Indianapolis, IN). All reactions were performed in triplicate and normalized to *βactin*. No amplification was observed in the negative control PCR reactions using non-reverse transcribed RNA. Relative message levels were calculated using the ΔΔCT method and 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 to 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 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 90-100% were utilized. Primer sequences and their annealing temperatures are listed in Supplemental Table 1.

Immunohistochemistry

Kidneys were removed from healthy C57BL/6 (16- to 18-week-old), nephritic MRL/lpr (16- to 18-week-old) and nephritic NZM2410 (28-to 34-week-old) mice, flash frozen, embedded in OCT and sectioned at 4-5 micron 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 Inc., Santa Cruz, CA). 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 minutes and counterstained in

Carazzi's Hematoxylin for 45 seconds. Tissues were dehydrated and cleared in ethanol gradients, xylene and mounted with Cytoseal 60 (Thermo Fisher, 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 20-40X. 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 glomeruli.

Immunofluorescence

NEU1, NEU3 and HA-IgG were detected in primary MCs using immunolabeling techniques. Cells were allowed to adhere and grow in sterile 8-chamber slide (Fisher, Pittsburgh, PA) for a period of 24 hours. 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 expressions with respect to IgG deposition on renal sections were 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 Inc.). NEU1 and NEU3 were detected with Alexa Fluor 488 conjugated chicken anti-rabbit (Thermo Fisher) 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

Neuraminidase (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 pre-nephritic (8-9wks of age) and nephritic (23-26wks of age) NZM2410 mice. Total protein was determined using the microBCA assay (Thermoscientific, Rockford, IL). Results are reported as relative fluorescence units (RFU)/min/mg 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 was collected and the cells were washed with 37°C warmed sterile filtered Tris Buffered Saline pH 7.4 (TBS). Cells were then incubated at 37°C with 15 μM 2' (4-Methylumbelliferyl) - ∝-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 was 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, Inc., La Jolla, CA). The association between NEU activity between mice with early and late disease (Fig. 1A) or between MCs treated with vehicle and HA-IgG at different time points post-treatment (Fig. 4B) were evaluated using a series of 2-sample t-tests. Adjustment for multiple comparisons for the p-values in the t-tests were calculated using Welch's correction due to unequal variance for Fig. 1A and the Holm-Sidak test for Fig. 4B. The association between IL-6 or MCP-1 levels with HA-IgG concentration and treatment group (empty, NEU1, NEU3) were evaluated using 2-way ANOVA model including effects for HA-IgG dose and treatment group (Fig. 2B 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, 4A, 4C, 4D, 5C, 5D, and 6) or one dose of HA-IgG over time (Figs. 5A and 5B) 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 posthoc 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 increases in the kidneys of nephritic MRL/lpr lupus mice and parallels increases in LacCer and GlcCer (due to catabolism of gangliosides by NEUs) in the kidneys and urine (34). To determine if increased NEU activity in the kidneys may be a common phenomenon in lupus nephritis (LN), we measured NEU activity in mice at an early disease stage (8-10 weeks old) and mid/late disease stage (23-28 weeks old) NZM2410 mice. In agreement with results observed in the MRL/lpr lupus strain, NEU activity is significantly increased in the kidneys of mid/late disease stage compared to early disease stage (Fig. 1A). Of the eight 23-28 week-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 week-old mice were negative for protein or had only trace amounts of protein in their urine. Although some of the 23-28 week-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 prior to 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). Therefore, we examined expression of NEU1 and NEU3 in the kidneys of early disease and late disease MRL/lpr and NZM2410 mice by immunohistochemistry to determine the renal cell types expressing these factors. Both NEU1 and NEU3 are expressed most highly in the glomeruli and are detectable

in all glomerular cell types, including mesangial cells (MCs) (Fig. 1B and 1C). Semi-quantitation 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 show 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 non-specific 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. Based on 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 analogues (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 pro-inflammatory cytokines and chemokines were determined using a pathway focused RT^2 profiler PCR^{TM} Array (SA Biosciences). Approximately 50 pro-inflammatory cytokines and chemokines were upregulated (two-fold or greater compared to vehicle treated) in the GSL treated cells (Supplemental Table S1). Twelve of the 50 genes were identified as kidney-relevant pro-inflammatory factors using Ingenuity Pathways Analysis software. Eleven of those genes were verified to be increased 2-fold or greater (Fig. 2A) using independently designed and optimized primers (Supplemental Table S2). These results suggest that elevated GSL catabolism in MCs is sufficient to induce production of inflammatory cytokines that play a major role in renal infiltration of immune cells.

Il-6 was one of the more highly upregulated cytokine genes in Fig. 2A with a more than four-fold increase in response to LacCer/GlcCer compared to 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. 1B and 1C, black bars). However, overexpressing NEU1 or NEU3 resulted in a significant HA-IgG dose-dependent increase in IL-6 (Fig. 2B), but not MCP-1 (Fig. 2C). Interestingly, both NEU1 and NEU3 significantly and dose-dependently increased the production of IL-6 in the absence of external stimulation by HA-IgG (Fig. 3A and 3C). MCP-1 was significantly increased only when transfected with the highest concentrations of NEU1 and NEU3 (Fig. 3B and 3D). 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 the protein PPCA (8, 49). Since PPCA was not co-overexpressed 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 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 if NEU activity plays a role in activation of lupus prone MCs, we generated primary MC lines from kidneys of early disease stage (pre-nephritic) lupus prone MRL/lpr mice. MCs were treated with different concentrations of HA-IgG (0.5 – 67.5 μg/ml) for 24 h and *Neu1* and *Neu3* mRNA expression and NEU activity were measured. HA-IgG significantly increased *Neu1* mRNA expression at the highest concentrations (22.5 and 67.5 μg/ml) (Fig. 4A). Although *Neu3* mRNA also appears to be increasing in response to the higher concentrations of HA-IgG, the increases were not significant. *Neu3* message levels were barely detectable and 2-5-fold lower relative to *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 1-5 min compared with vehicle treated cells (Fig. 4B). The increase in *Neu1* message levels and NEU activity corresponded with a significant dose-dependent secretion of IL-6 (Fig. 4C) and MCP-1 (Fig. 4D). Similar dose-dependent increases of IL-6 and MCP-1 were observed 24 h and 48 h after HA-IgG stimulation with the 24 h data presented in Fig. 4. These results suggest that increased NEU activity may be due largely to increased NEU activity following HA-IgG stimulation of lupus prone MCs. Together, the results in Figs. 2-4 suggest that NEU activity may mediate IL-6 production following activation of lupus prone MCs.

IL-6 production in HA-IgG stimulated MRL/lpr lupus MCs is mediated by NEU activity

To determine if 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 6-48 h compared to vehicle treatment (Fig. 5A). For MCP-1, a gradual increase in production beginning at 3 h and continuing over 48 h was observed (Fig. 5B). Therefore, we examined the effect of oseltamivir phosphate (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. 5C and 5D) 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%. Since OP inhibits all NEU activity, siRNA for Neu1 was used to knock down Neu1 message levels in the MRL/lpr MCs. Neu1 siRNA reduced Neu1 mRNA levels by ~40% (Fig. 5E) and resulted in a significant reduction in IL-6 production (Fig. 5F). Neu3 message levels were also measured and remained at nearly undetectable levels regardless of treatment. 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 activity in IL-6 and MCP-1 production in the primary MCs when stimulated with serum from 18-week-old nephritic MRL/lpr mice, which

contains high levels of IgG (53). IL-6 production by the MCs increased as the percentage of MRL/lpr serum increased, which was significant with 10% lupus serum (Fig. 6A). Serum from 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. 6B). Importantly, as with HA-IgG stimulation, OP significantly suppressed lupus serum-stimulated IL-6 production in a dose-dependent manner 3 h (Fig. 6C) and 6 h (data not shown) after stimulation. These results indicate that IL-6 production of lupus prone MCs following activation by lupus serum is mediated by NEU activity. Combined with the results above with HA-IgG, it is likely that NEU may specifically mediate IL-6 production by lupus prone MCs activated by circulating IgG-containing immune complexes.

Expression of NEU1 and NEU3 overlap with IgG deposits in MRL/lpr MC cultures and renal sections

NEU3 is typically located in the plasma membrane and although NEU1 is largely located in the lysosome, it is also found at the cell surface in many cell types (23, 25, 33). Therefore, to determine if NEU1 and/or NEU3 may be co-localizing with HA-IgG binding on the surface of the MRL/lpr MCs, we performed immunofluorescence. As demonstrated previously in B6 MCs (45), HA-IgG binds to the surface of MRL/lpr MCs (Fig. 7B). Staining for both HA-IgG and either NEU1 (Fig. 7C) or NEU3 (Fig. 7D) shows that HA-IgG binding overlaps with cell surface staining for NEU1 and NEU3. We then examined localization of NEU1 and NEU3 with respect to IgG deposition in renal sections from nephritic MRL/lpr mice. Similar to the overlap observed in the MC cultures, overlap was observed between IgG deposits (red) and either NEU1 (Fig. 1E) or NEU3 (Fig. 7F) (green) in the glomeruli specifically in the 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 to their non-nephritic counterparts (34). We have now demonstrated that NEU activity in nephritic

NZM2410 lupus mice also is significantly increased compared to pre-nephritic 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 if the increased NEU activity detected in the MRL/lpr(34) and NZM2410 (Fig. 1A) kidneys is a result of increased NEU1/NEU3 expression and/or activation.

MCs occupy the central stalk of the glomerulus and interact with endothelial cells and podocytes. It gives structural support and contributes to the regulation of glomerular filtration, occupying approximately 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 pro-inflammatory 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 the due to infiltrating inflammatory cells in LN patients. However, based on 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.

Our studies here 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 oseltamivir phosphate (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 and the more pronounced effect on IL-6 production when NEU activity is blocked by OP suggests that GSL catabolism (NEU activity) directly impacts IL-6 production in activated lupus prone MCs. The effect on MCP-1 production may be a secondary effect due to IL-6 autocrine effects.

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 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 approximately 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 mM 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. While 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 prior to HA-IgG activation. NEU1 was shown to have broader substrate specificity compared to 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). Since loss of sialic acids, and specifically loss of ganglioside GM3, increases MC proliferation (20), we speculate that inhibiting NEU activity prior to 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 non-permeablized 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 response to immune complex deposition in LN (5). MCs express FcRs that bind IgG and HA-IgG was shown to co-localize 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 co-localize with TLR2 and TLR4 on the surface of macrophages and dendritic cells and mediate signaling (1, 4, 10). Although TLRs 1-4 are expressed by cultured MRL/lpr MCs, only the endosomal TLR3 was detected in MCs on renal sections from MRL/lpr mice (35). Alternatively, NEU activity may mediate IgG activation of MCs through 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. Ongoing studies are focused on identifying the mechanisms by which NEU mediates IL-6 signaling in MCs.

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 IgG deposition. 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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Figures

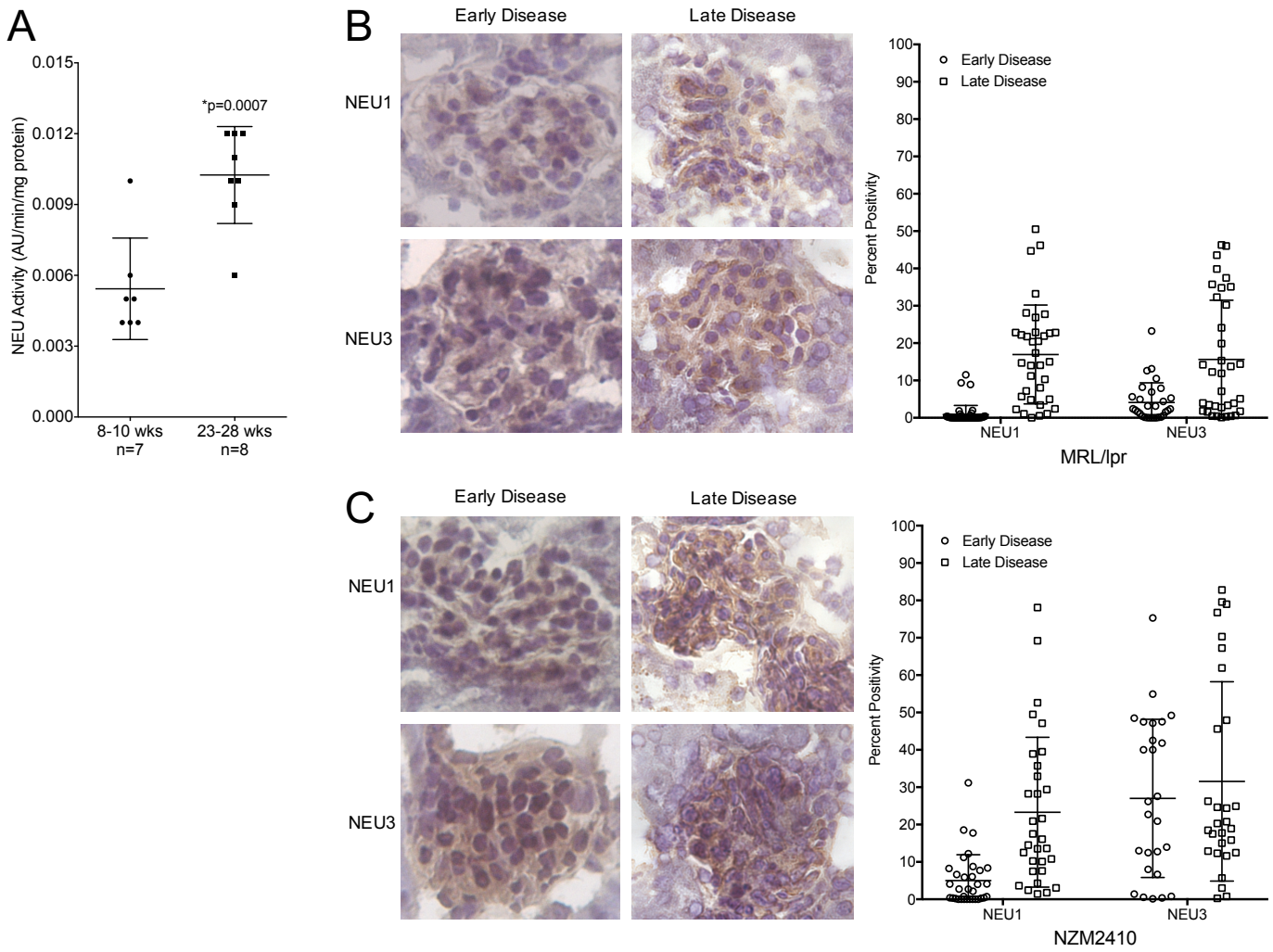

Figure 1. Renal NEU activity is elevated and NEU1 and NEU3 are highly expressed in MCs of nephritic lupus mice. A) NEU activity was measured in kidney homogenates from 8-10 week-old pre-nephritic and 23-28 week-old nephritic NZM2410 mice. P-value was calculated as described in Materials and Methods. Immunohistochemistry analyses and semi-quantitative 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.

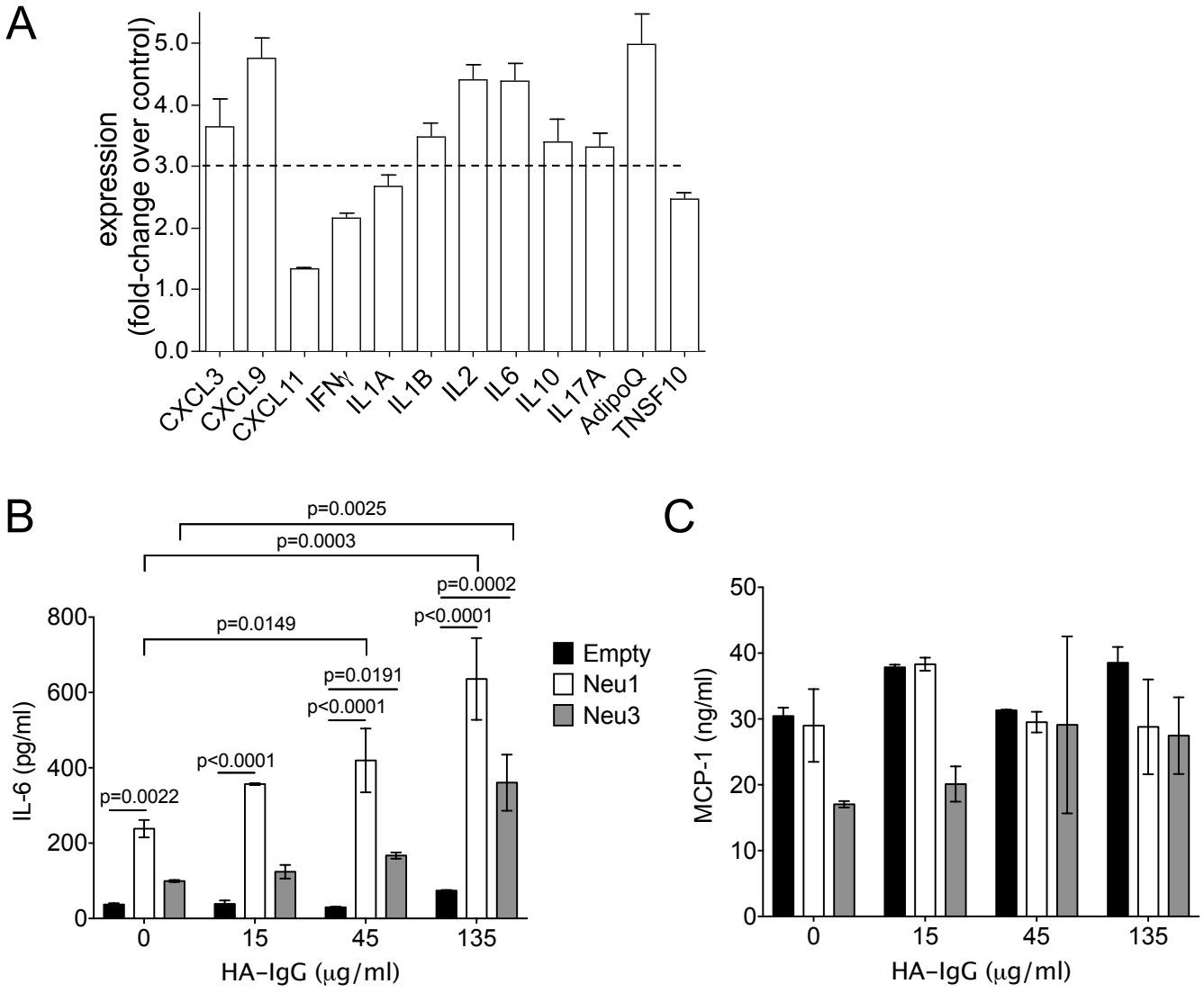

Figure 2. GSL catabolism upregulates *Il-6* gene expression and IL-6 production by MES13 MCs. A) MES13 MCs were incubated with LacCer and GlcCer, mRNA isolated, and cytokine gene expression measured by real-time PCR in reverse-transcribed mRNA. Up-regulated genes are presented as fold increase over vehicle 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.

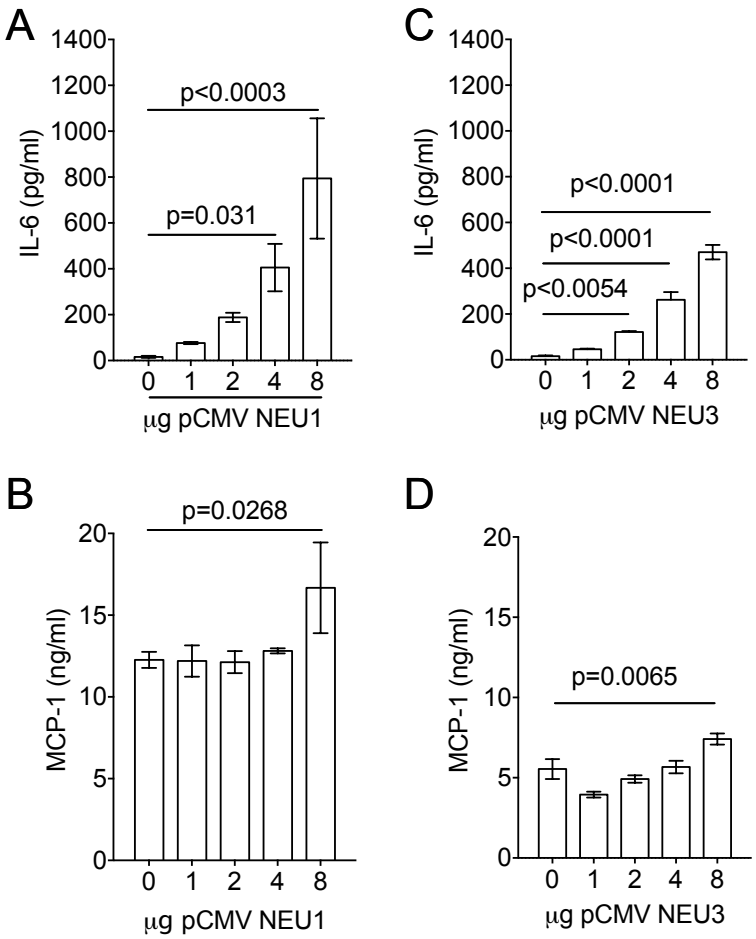

Figure 3. MES13 MCs produce IL-6 in a dose-dependent manner in response to NEU1 or NEU3 overexpression in the absence of stimulation. MES13 mouse MCs were transfected with increasing concentrations of a NEU1 (A and B) or NEU3 (C and D) expression plasmid and media collected 24 h (not shown) and 48 h (presented) post-transfection. 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, $p<0.0001$. Posthoc p-values are provided on the graphs.

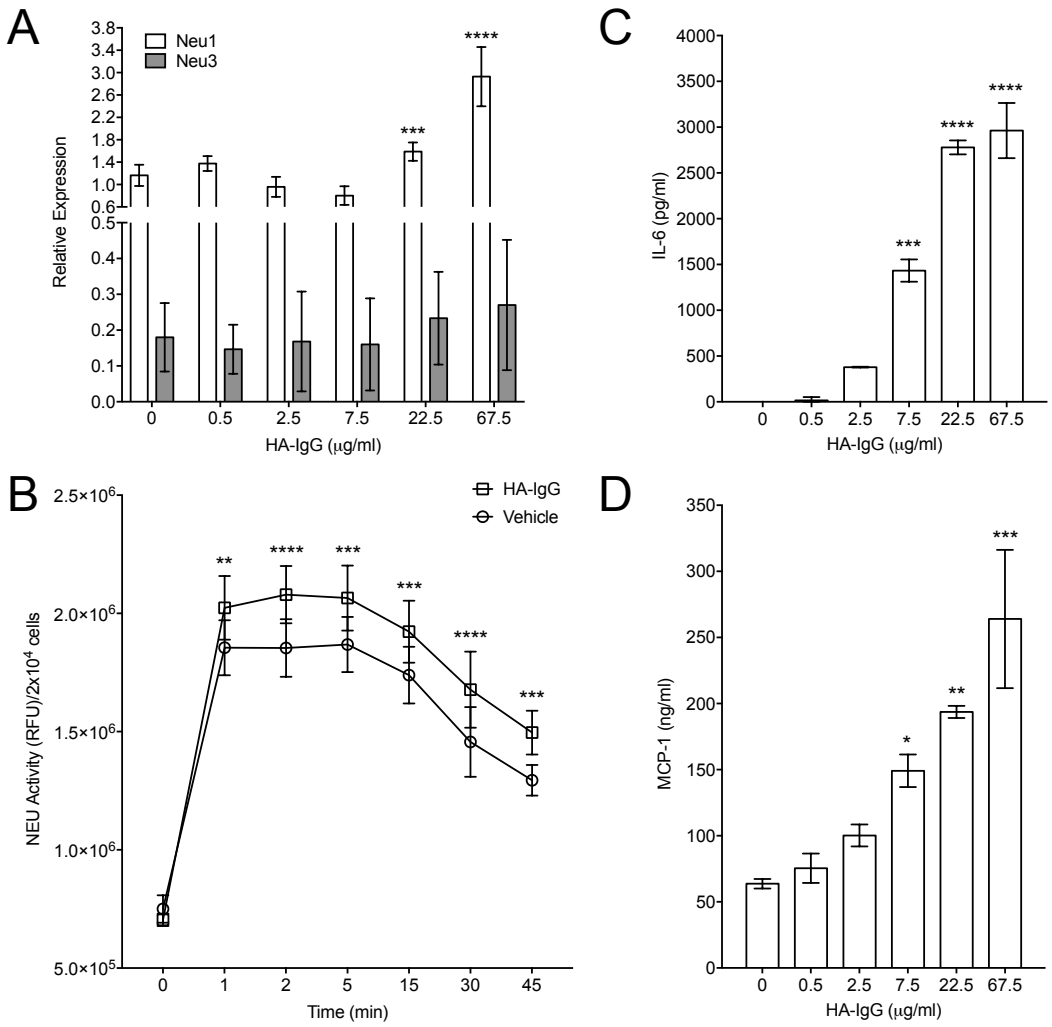

Figure 4. HA-IgG stimulation of primary MRL/lpr lupus prone MCs increases *Neu1* message levels, NEU activity, IL-6 and MCP-1 production. Primary MRL/lpr MCs were grown out of glomeruli isolated from pre-nephritic mice and stimulated with HA-IgG. A) Cells were treated with increasing concentrations of 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 RTPCR. 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, posthoc 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.

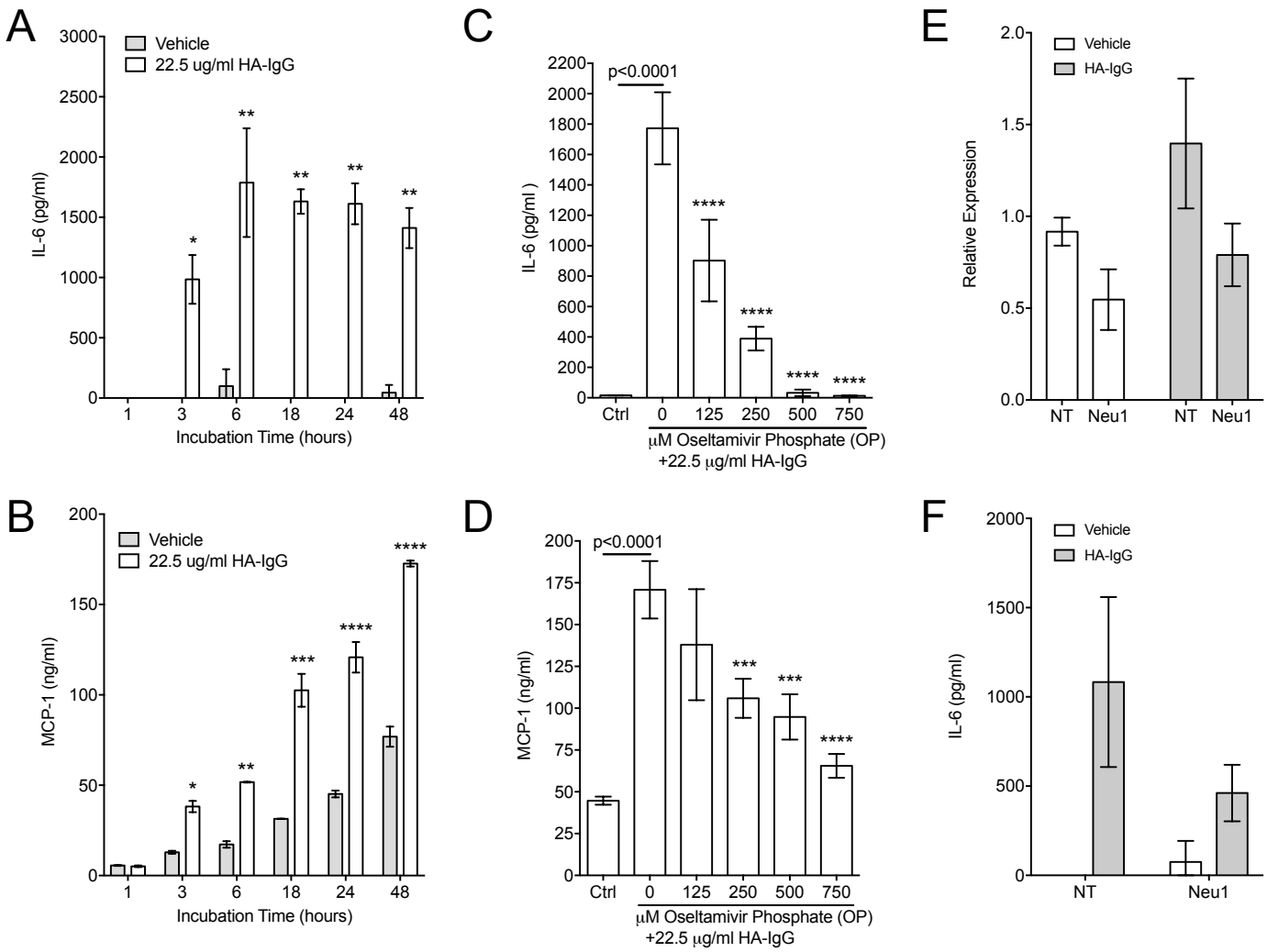

Figure 5. Inhibiting NEU activity blocks HA-IgG-stimulated IL-6 and MCP-1 production by MRL/lpr lupus prone MCs. A and B) Primary MRL/lpr MCs were treated with 22.5 μg/ml of HA-IgG over time. IL-6 (A) and MCP-1 (B) levels were measured in media by ELISA and normalized to total protein. C and D) Primary 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. Cells were transfected with Neu1 or non-target 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, posthoc 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.

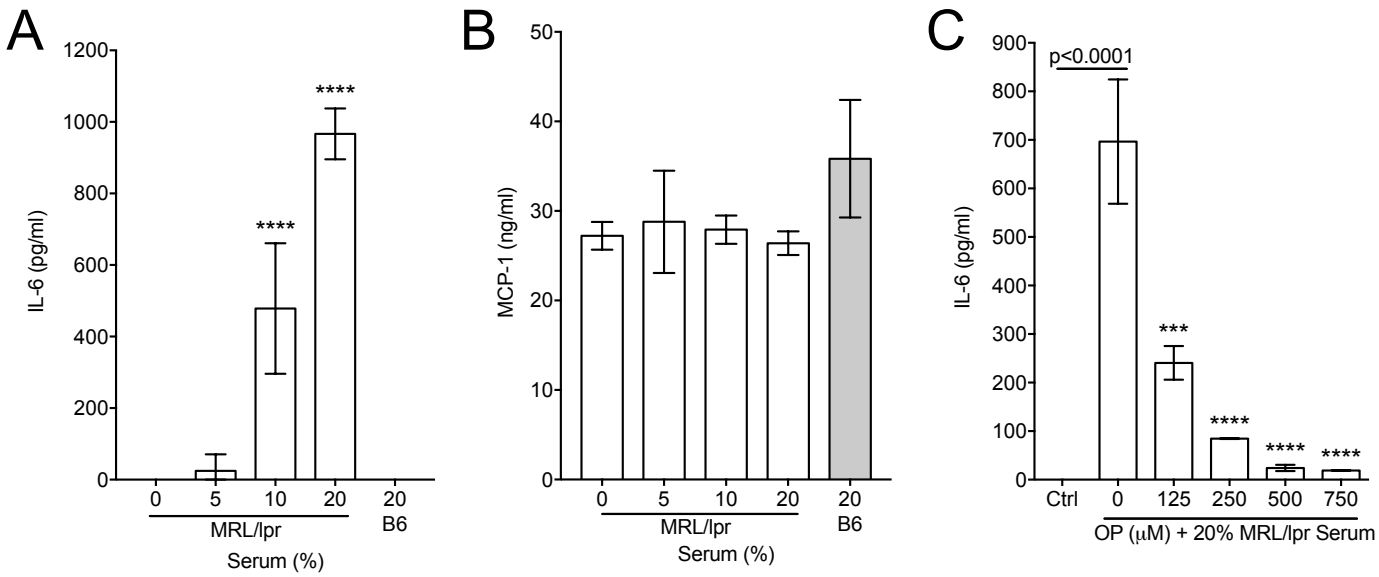

Figure 6. Inhibiting NEU activity blocks lupus serum-stimulated IL-6 production by MRL/lpr lupus prone MCs. Primary MRL/lpr MCs were treated with increasing concentrations of MRL/lpr serum or B6 serum for 24 h. IL-6 (A) and MCP-1 (B) levels in media were measured by ELISA and normalized to total protein. C) MRL/lpr MCs were cultured with increasing concentrations of oseltamivir phosphate (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, posthoc 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.

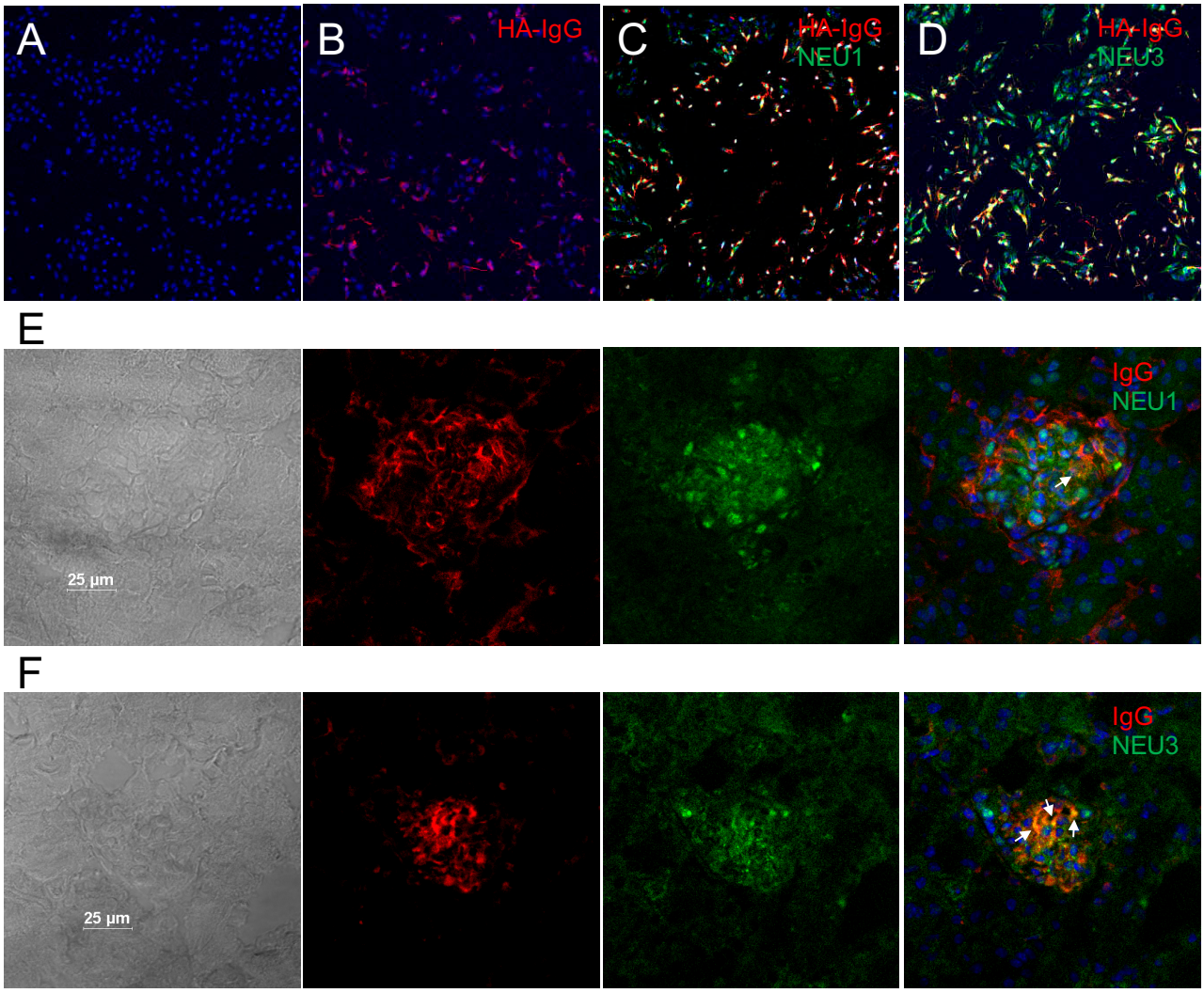

Figure 7. NEU1 and NEU3 expression overlaps with IgG deposits on the surface of MRL/lpr lupus prone MCs and in the glomeruli of nephritic MRL/lpr mice. MRL/lpr lupus prone MCs were fixed (but not permeablized) and stained with isotype (A) or HA-IgG for 1 h followed by Alexa Fluor 594 goat anti-mouse IgG secondary antibody (B, C and 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). White arrows indicate areas of overlap, which are largely mesangial, between IgG and NEU1 or NEU3 in the composite images.