Received: 28 July 2021 | Revised: 9 February 2022 | Accepted: 17 March 2022

DOI: 10.1096/fj.202101218R

THE FASEB JOURNAL

RESEARCH ARTICLE

# The Janus-like role of neuraminidase isoenzymes in inflammation

**Md. Amran Howlader**[1] | **Ekaterina P. Demina**[2] | **Suzanne Samarani**[3] | **Tianlin Guo**[1] | **Antoine Caillon**[2] | **Ali Ahmad**[3] | **Alexey V. Pshezhetsky**[2,4] | **Christopher W. Cairo**[1]

[1]Department of Chemistry, University of Alberta, Edmonton, Alberta, Canada

[2]Division of Medical Genetics, Sainte-Justine Hospital Research Center, University of Montreal, Montreal, Quebec, Canada

[3]Department of Microbiology, Infectious Diseases & Immunology, Sainte-Justine Hospital Research Center, University of Montreal, Montreal, Quebec, Canada

[4]Department of Anatomy and Cell Biology, McGill University, Montreal, Quebec, Canada

**Correspondence**

Alexey V. Pshezhetsky, Sainte-Justine Hospital Research Center, Division of Medical Genetics, University of Montreal, Montreal, Quebec, Canada.
Email: alexei.pchejetski@umontreal.ca

Christopher W. Cairo, Department of Chemistry, University of Alberta, Edmonton, Alberta, T6G 2G2, Canada.
Email: ccairo@ualberta.ca

**Funding information**

This work was supported by a GlycoNet student travel grant (MAH), GlycoNet collaborative team grants (CD-2: CWC and AVP; ID-01: CWC, AVP, and AA), and the Canadian Institutes of Health Research (Grant PJT-148863, CWC and AVP)

## Abstract

The processes of activation, extravasation, and migration of immune cells to a site are early and essential steps in the induction of an acute inflammatory response. These events are an essential part of the inflammatory cascade, which involves multiple regulatory steps. Using a murine air pouch model of inflammation with LPS as an inflammation inducer, we demonstrate that isoenzymes of the neuraminidase family (NEU1, 3, and 4) play essential roles in these processes by acting as positive or negative regulators of leukocyte infiltration. In genetically knocked-out (KO) mice for different NEU genes (*Neu1* KO, *Neu3* KO, *Neu4* KO, and *Neu3*/*4* double KO mice) with LPS-induced air pouch inflammation, leukocytes at the site of inflammation were counted, and the inflamed tissue was analyzed using immunohistochemistry. Our data show that leukocyte recruitment was decreased in NEU1- and NEU3-deficient mice, while it was increased in NEU4-deficient animals. Consistent with these results, systemic as well as pouch exudate levels of pro-inflammatory cytokines were reduced in *Neu1* and increased in *Neu4* KO mice. Pharmacological inhibitors specific for NEU1, NEU3, and NEU4 isoforms also affected leukocyte recruitment. Together our data demonstrate that NEU isoenzymes have distinct—and even opposing—effects on leukocyte recruitment, and therefore warrant further investigation to determine their mechanisms and importance as regulators of the inflammatory cascade.

**KEYWORDS**

inflammation, leukocytes, neuraminidase, sialic acid, sialidase

**Abbreviations:** BMDM, bone marrow–derived macrophage; BW, body weight; DKO, double knock out; IN1, inhibitor of NEU1 (CG14600); IN3, inhibitor of NEU3 (CG22600); IN4, inhibitor of NEU4 (CY16600); KO, knock out; LPS, lipopolysaccharide; MOs, monocytes; MΦ, macrophages; NE, neutrophil; NEU1, neuraminidase 1; NEU3, neuraminidase 3; NEU4, neuraminidase 4; NK, natural killer cells; WT, wild type.

Md. Amran Howlader and Ekaterina P. Demina contributed equally to this work.

## 1 | INTRODUCTION

The induction of the inflammatory cascade by exogenous or endogenous stimuli culminates in acute inflammation: a host protective response that tends to neutralize the stimulus, and maintains tissue homeostasis and integrity. However, when inflammation becomes uncontrolled and long-lasting, it contributes to the pathogenesis of chronic inflammatory diseases such as sepsis, diabetes, atherosclerosis, and cardiovascular disease. The cascade begins with activation and trafficking of leukocytes to the site of inflammation.[1] It provides a rapid response to an infection by regulating permeability of the vascular compartment, activation of endothelial cells, tethering and adhesion of leukocytes to the vascular endothelium, and subsequent extravasation to the site of inflammation as well as activation of macrophages, platelets, complement, and clotting factors. Acute inflammation is triggered by recognition of pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS) of gram negative bacteria, by one or more pattern recognition receptors (PRRs) of the host cells (e.g., toll-like receptors, TLRs).[2] For example, LPS is recognized by TLR-4 leading to initiation of the inflammatory cascade and leukocyte recruitment.[3] The glycosylation state and activity of cellular receptors, including TLR-4, is known to be regulated by endogenous neuraminidase enzymes (NEU or sialidases).[4–6] Notably, there are four NEU isoenzymes, each with distinct tissue expression, sub-cellular localization, and role in inflammation and pathogenesis.[7] These enzymes remove sialic acid from the termini of glycoproteins, glycolipids and oligosaccharides in a substrate- and linkage-specific manner. Increased endogenous neuraminidase activity, accompanied by leukocyte activation, is known to play a role in the infiltration of leukocytes to the site of inflammation; however, the precise role of individual NEU isoenzymes in leukocyte recruitment has not been investigated in a systematic fashion.[8,9]

As a terminal residue of many glycans, *N*-acetylneuraminic acid (Neu5Ac, or sialic acid) is known to be important in multiple steps of the inflammatory cascade. Selectins, expressed by immune cells as well as by vascular endothelial cells (VEC), mediate leukocyte capture and rolling and require minimal tetrasaccharide epitopes containing Neu5Ac residues (sialyl-Lewis$^X$; sLe$^X$ or CD15) for binding with their ligands (e.g., PSGL-1, CD44).[10] Endogenous NEU are able to desialylate these epitopes, thus disrupting selectin-ligand interactions.[9] The firm adhesion step of the cascade is mediated by activated leukocyte β2 integrins, LFA-1 (CD11a/CD18) and MAC-1 (CD11b/CD18), which bind immunoglobulin-like adhesion molecules (ICAM-1 and ICAM-2) expressed by activated VEC. Activation epitopes of β2 integrins were reported to be unmasked by endogenous NEU activity,[8,11] and NEU3 has been shown to alter LFA-1/ICAM-1 interactions.[12] NEU enzymes can increase adhesion of polymorphonuclear (PMN) leukocytes to endothelium and also increase their migration through the endothelium to the site of inflammation.[13,14] They may also unmask epitopes in β1 integrins, VLA4 (CD49d/CD29) and VLA5 (CD49e/CD29), which are expressed on activated leukocytes and bind with VCAM-1 and fibronectin (FN).[8,11] Activated leukocytes have been shown to increase production of inflammatory cytokines such as TNF-α and IFN-γ through the action of NEU1 or NEU3.[15] Furthermore, the effects of cytokine production (e.g., IL-6, IL-12p40, and TNF-α) in leukocytes (e.g., dendritic cells) have also been reported to be regulated by NEU1 and NEU3 activity.[16] Notably, NEU1 has been shown to induce phagocytosis in macrophages by the activation of Fc-γ receptors.[17] Together, these findings suggest that endogenous NEU activity is involved at multiple points along the inflammatory cascade.

In the current work, to test the hypothesis that individual NEU isoenzymes have different effects on inflammation and to address specific roles of these enzymes in recruitment of leukocytes to the site of inflammation, we used a murine 6-day air pouch model of LPS-induced acute inflammation[18] in *Neu1*, *Neu3*, *Neu4* KO and WT mice (in an identical C57Bl6 genetic background). In previous studies, we demonstrated that *Neu3* and *Neu4* enzymes have similar substrate specificity and can complement each other[19]; therefore, we also tested *Neu3*/*4* double KO (DKO) mice, which are deficient in both enzymes.[20] We observed that NEU isoenzymes drastically modulated the inflammatory response to LPS, with NEU1 and NEU3 acting as positive regulators, and NEU4 acting as a negative regulator, of the response. These results reveal that NEU isoenzymes have important and distinct roles in the regulation of immune cell migration to the site of inflammation as well as consequent inflammation. Moreover, our findings suggest that NEU isoenzymes play roles in immune response and may be targeted to modulate pathogenic inflammation in human disease.

## 2 | MATERIALS AND METHODS

### 2.1 | Animal models

C57BL6 mice (aged 3–4 months) were used as wild-type control. *Neu1* KO, *Neu3* KO, *Neu4* KO, and *Neu3*/*4* DKO mice were as reported previously.[20] Mice were housed in an enriched environment with continuous access to food and water, under constant temperature and humidity,

and on a 12 h light/dark cycle. The experiments involving animals were approved by the Animal Care and Use Committee of the CHU Ste-Justine Research Center (protocol #710). WT and *Neu1* KO groups contained both male and female mice, whereas other groups contained only male mice.

## 2.2 | Sources of reagents and antibodies

Compounds **IN1** (CG14600), **IN3** (CG22600), and **IN4** (CY16600) were prepared as previously reported.[21–24] Stock solutions for these compounds were made using sterile saline (0.90% NaCl). An LPS isolated from *E. coli* O55:B5 that does not contain sialic acid was purchased from Sigma Aldrich (cat# L2880).[25] Anti-mouse/human CD11b FITC (Clone M1/70), anti-mouse CD45 Alexa Fluor 700 (Clone 30-F11), anti-mouse/human CD45R/B220 PerCP (Clone RA3-6B2), anti-mouse 49b PE (Clone DX5), anti-mouse F4/80 APC (Clone BM8), anti-mouse Ly-6G PE/Cyanine 7 (Clone 1A8), anti-mouse Ly-6C Brilliant violet 421 (Clone HK1.4), and anti-mouse CD115 APC/Cyanine 7 (Clone AFS98) were purchased from Biolegends, USA.

## 2.3 | Air pouch model of acute inflammation

The air pouch inflammation model was previously described and used here with slight modifications.[26–28] Briefly, mice (6–8 weeks, male or female) were randomly separated into control and experimental groups. Hair was removed at the dorsal area (5 × 2 cm) 2 days prior to commencing the experiment, and Vaseline was applied on the nude skin area to alleviate discomfort. Animals were monitored for abnormal behavior and skin injuries daily. On days 3 and 6, mice were anesthetized under isoflurane and 3 ml of sterilized air (passed through a 0.2-μm filter) was injected subcutaneously into the back of the mice using a 26-gauge needle. On day 8, LPS (1 μg in 1 ml of sterile PBS) or PBS alone (as control) was injected into the air pouches. To investigate the impact of NEU inhibitors on the air pouch inflammation, intraperitoneal injections of NEU-specific inhibitors were made on days 6, 7, and 8 (200 μl of sterile saline containing inhibitors at 1 mg/kg dose). Saline was injected as control for NEU inhibitors. Nine hours after infusion of LPS, mice were sacrificed using phenobarbital overdose (150 mg/kg BW). Then, air pouches were washed twice with 1.0 ml of HBSS containing 10 mM EDTA, and the washes (exudates) were collected and centrifuged at 100*g* for 10 min at room temperature. The supernatants were collected and frozen for later analysis. The cells in the exudates were resuspended in 1 ml of HBSS-EDTA and counted by haemocytometer or used in flow cytometry analysis.

## 2.4 | Flow cytometry analysis of immune cells

Subpopulations of the cells isolated from the air pouch exudates were analyzed by flow cytometry as previously described.[29] Dead cells were stained with Aqua blue (Thermo Fisher Scientific) and Fc Receptors (FcR) were blocked using mouse IgG. After washing with PBS (containing 2% FBS), cells were incubated on ice for 30 min with fluorophore-conjugated antibodies as a mixture: CD49b PE, CD45 AF700, CD115 APC/CY7, Ly-6C BV421, Ly-6G PE/CY7, F4/80 APC, B220 PerCP, CD11b FITC, CD3 FITC, and CD11c APC. The stained cells were washed twice with PBS, resuspended in 2% paraformaldehyde and analyzed by flow cytometry using BD LSRII Fortessa. Single fluorochromes were used for compensation (elimination of spectral overlap) and for setting of gates using a minus one method. Data were analyzed by Diva and FlowJo. Dead cells and doublets were eliminated from the analyses.

## 2.5 | Immunohistochemistry protocol and cell counts

The pouch tissues (skin walls) were embedded in Tissue Tek OCT from Sakura, and 4 μm thick sections were cut and mounted on glass slides for immunohistochemistry (IHC). The slides were stained with hematoxylin and eosin (H&E), and then imaged using a ZEISS Axio Scan Z1. Regions of the IHC slides were identified as three major regions: epidermis, dermis, and underlying muscle layer. The H&E staining revealed leukocytes present in each region. The number of leukocytes were counted with ImageJ using at least three randomly chosen fields of each region. The skin sections of at least three mice from each group were investigated.

## 2.6 | Cytokine analysis using ProcartaPlex immunoassays

Levels of cytokines in mouse plasma and exudates were determined using ProcartaPlex immunoassays (Perkin Elmer, USA) with a customized panel of 21 cytokines according to the manufacturer's protocol. Briefly, plasma and exudate samples were diluted in the sample diluent and incubated with capture antibody-coupled magnetic beads on a shaker at 4°C overnight. Unbound sample contents were removed and wells were washed thrice. Then, biotinylated secondary antibody was added, and

incubated on a shaker in the dark at room temperature for 30 min. Each captured cytokine was detected by addition of streptavidin-phycoerythrin and fluorescence was measured using the Autoplex Analyser CS1000 system (Perkin Elmer, Waltham, MA).

### 2.7 | Macrophage preparation and chemotaxis trans-migration studies

Monocytes (MOs) were isolated from the bone marrow of mice and differentiated in vitro into macrophages (MΦ) by incubation in complete DMEM (cDMEM; DMEM supplemented with 10% heat-inactivated FBS, 10% conditioned media supernatant from L-929 fibroblast cell cultures, 1% penicillin-streptomycin, 0.01M HEPES buffer and 1 mM sodium pyruvate). They were cultured for 7 days in a T75 flask until they reached 80% confluency. The differentiated macrophages were then scraped off and used for the transmigration assay adapted from a previous study.[30] Briefly, an 8-μm-pore size Transwell plate (Costar, Fischer Sci, USA) was used after coating the Transwell membrane with FN (50 μg/mL in PBS) for 1 h, and then blocking it with bovine serum albumin for 30 min. The bottom chamber of the Transwell was filled with PBS containing a chemoattractant, Monocyte Chemoattractant Protein-1 (MCP-1, 20 ng per well; Thermofisher Scientific, USA). Macrophages ($5 \times 10^4$ cells in PBS) isolated from the genotypic mice were gently added to the top chamber of the Transwell and incubated for 5 h at 37°C with 5% $CO_2$. Subsequently, the upper chamber was removed, wiped gently with a Q-tip and the Transwell membrane was stained with Kwik-Diff stain (Fisher Scientific). Cells that had infiltrated the membrane were then imaged using Zeiss COLIBRI fast LED imaging and optical sectioning microscope using 10X lens under bright field. The images were analyzed using Zen black software (Zeiss).

### 2.8 | Statistical analyses

Pearson correlation coefficients, r and P values between the concentrations of cytokines in plasma and exudates and cell counts in exudates of *Neu1* KO, *Neu4* KO and WT mice treated with PBS or LPS, were calculated using SigmaPlot version 13 (Systat Software, San Jose, CA). The correlations with $p < .05$ were considered statistically significant. Data means were compared using one or two-way ANOVA with Dunnett's multiple comparison test. The data for cytokines that showed statistically significant correlations were tested for randomness in at least one group of mice and were visualized with correlograms.

## 3 | RESULTS

### 3.1 | Inflammatory response to lipopolysaccharide reveals involvement of neuraminidase enzymes in the recruitment of leukocytes

We used the murine air pouch model to investigate the effect of individual neuraminidase enzymes on LPS-induced inflammation.[18] The air pouch was created by subcutaneous injection of sterile air into the lower dorsal region of WT and *Neu1* KO, *Neu3* KO, *Neu4* KO, and *Neu3/4* DKO mice (see Materials & Methods). The pouch was injected with either 1 μg LPS in 1 ml of saline, to simulate a bacterial infection, or with 1 ml of saline (as control). After an incubation period of 9 h, the air pouch was washed with sterile saline to harvest cells infiltrated into the pouch exudate. The cells were then counted by flow cytometry (Figure 1 and Table 1). The differences in the pouch exudate cell counts between WT and the *Neu* KO mice at basal level were non-significant (Figure 1B). In contrast, significant differences in the cell numbers in the exudates were observed between LPS-treated WT and *Neu* KO mice (Figure 1C). In the *Neu1* KO mice, leukocyte counts were reduced to ~25% ($79 \pm 9 \times 10^4$ cells/pouch) of those in WT mice ($326 \pm 29 \times 10^4$ cells/pouch). A trend for reduction of leukocytes was also observed in the *Neu3* KO compared with the WT mice; however, the difference was not statistically significant. The *Neu4* KO ($1371 \pm 229 \times 10^4$ cells/pouch) and the *Neu3/4* DKO ($992 \pm 141 \times 10^4$ cells/pouch) animals demonstrated significant (three- to four-fold) increases in the cell numbers compared with the WT mice upon LPS treatment. These results suggested that LPS-stimulated leukocyte infiltration into the pouch was regulated positively by NEU1, and negatively by NEU4.

### 3.2 | Leukocyte subset response to lipopolysaccharide in neuraminidase-deficient animal models

To determine the influence of neuraminidases on leukocyte subsets found in the air pouch model, we stained cells from the exudate with cell type–specific antibodies and quantified them using flow cytometry. Cell counts were normalized to saline-treated WT control for the selected leukocyte subsets shown in Figure 2 (Figure SI1 and Table SI1 provide raw counts). We observed that the major populations found in the air pouch exudate after LPS treatment were MOs, neutrophils (NEs), natural killer cells (NK), and macrophages (MΦ). T and B lymphocyte counts were low for both saline-treated and LPS-treated animals, and no significant changes were

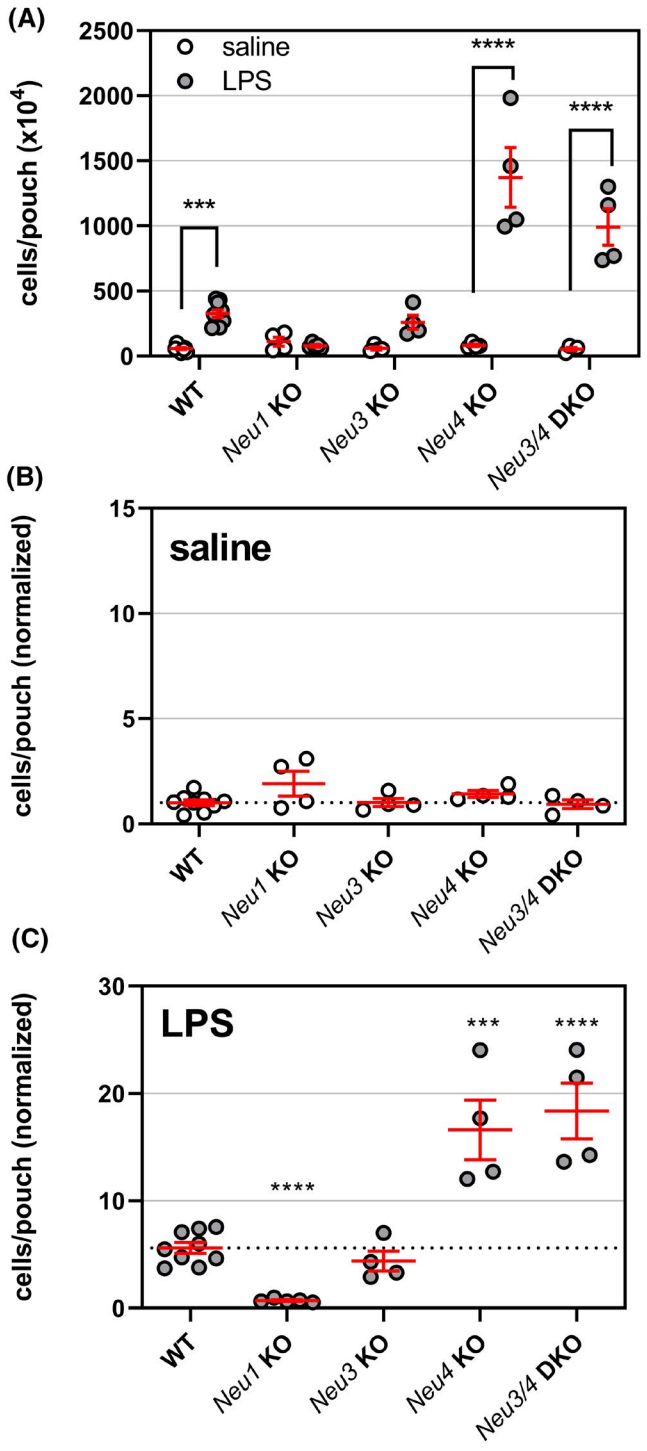



**FIGURE 1** Leukocyte counts from the air pouch exudates. The air pouch was formed after injection of sterile air for each mouse genotype. The pouch was injected with saline or LPS and incubated for 9 h. Thereafter, mice were sacrificed, the pouch exudate was collected, and cells were counted by flow cytometry. (A) Cell counts are presented for the saline (○) and LPS (●) treatments. (B) Cell counts for the saline treatment in the *Neu* KO mice are compared with those of WT mice ($58 \times 10^4$ cells/pouch; normalized to 1, dashed line). (C) Cell counts for the LPS treatment are compared with those of WT mice (normalized to WT saline treatment). Individual data points along with mean ± SEM are shown. For panel A, comparisons were made using two-way ANOVA following Dunnett's multiple comparison test; for panels B and C, comparisons were made using one-way ANOVA and Dunnett's *t*-test to compare groups to control. (***, $p \leq .005$; ****, $p \leq .0001$)

**TABLE 1** Leukocyte raw cell counts observed in the air pouch model

| | Saline (cells × $10^4$) | | | LPS (cells × $10^4$) | | |
|---|---|---|---|---|---|---|
| | Mean ± SEM | *p* | *N* | Mean ± SEM | *p* | *N* |
| WT C57BL6 | 58 ± 8 | | 9 | 326 ± 29 | | 9 |
| *Neu1* KO | 111 ± 34 | | 4 | 79 ± 9 | **** | 5 |
| *Neu3* KO | 59 ± 12 | | 4 | 259 ± 54 | | 4 |
| *Neu4* KO | 83 ± 9 | | 4 | 1371 ± 229 | *** | 4 |
| *Neu3/4* DKO | 54 ± 12 | | 4 | 992 ± 141 | **** | 4 |
| WT IN1 | 72 ± 16 | | 4 | 357 ± 111 | | 4 |
| WT IN3 | 100 ± 26 | | 4 | 189 ± 40 | | 4 |
| WT IN4 | 172 ± 43 | ** | 4 | 433 ± 49 | | 3 |

*Note:* Values were compared with their corresponding controls using a Student's *t*-test (**, $p \leq .01$; ***, $p \leq .005$; ****, $p \leq .0001$). Data are plotted in Figures 1A and 8A.

detected for any of NEU-deficient animals compared with WT mice (Table SI1). In contrast, cell type-specific differences between NEU-deficient and WT mice were revealed for other cell subsets. In particular, the *Neu1* KO mice had increased basal (saline treatment) MO and NE counts, and the *Neu3* KO animals had elevated basal MO counts. The *Neu4* KO mice had drastically elevated counts of MO, NE, and NK cells after LPS treatment (three- to ninefold increase compared with WT), as well as elevated basal MO counts (fivefold increase compared with WT). The *Neu3/4* DKO animals showed a moderate elevation of MO counts after LPS treatment, while the LPS-treated *Neu1* KO mice showed a trend for reduction of all cell counts compared with the WT mice. Together, these results are generally consistent with NEU4 acting as a negative regulator of MO, NE, and NK cell infiltration upon LPS stimulation.

## 3.3 | Immunohistochemistry detects different levels of leukocyte infiltration in NEU-deficient animals

To add further support to our conclusions regarding leukocyte infiltration, tissue samples from the air pouch walls were collected and analyzed by IHC (Figure 3, Table SI2 and Figure SI2). Sections of the tissues were stained with H&E reagent and relative numbers of leukocytes in

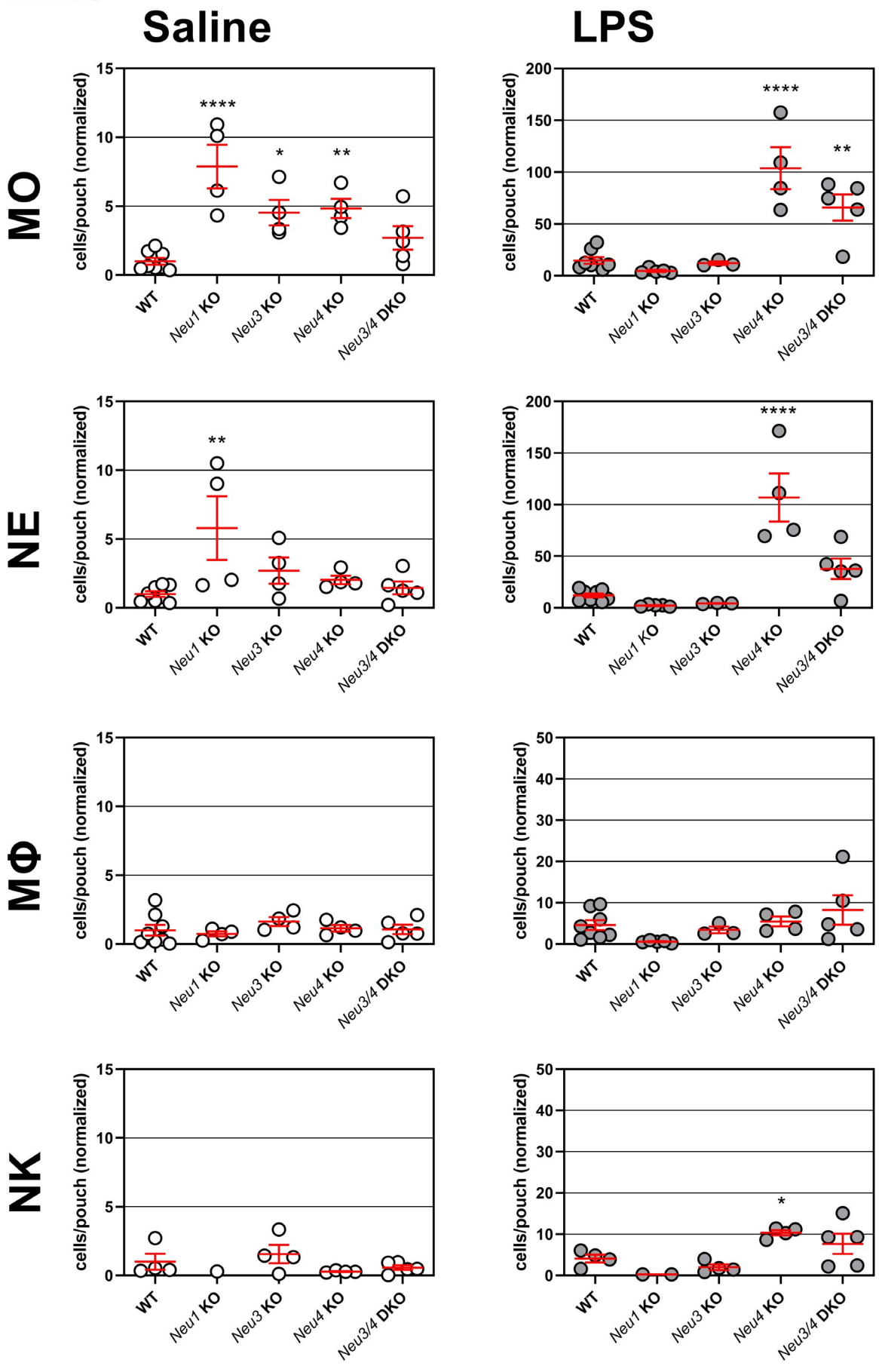

Saline
LPS
MO
NE
МФ
NK
cells/pouch (normalized)
WT
Neu1 KO
Neu3 KO
Neu4 KO
Neu3/4 DKO
****
*
**
****
**
**
****
*

**FIGURE 2** Changes in leukocyte populations in the air pouch model. Leukocytes collected from air pouch exudates from mice 9 h after saline (○), or LPS (●) treatment were counted by flow cytometry. The cell populations were identified after staining with marker-specific fluorochrome-conjugated antibodies as monocyte (MO), neutrophil (NE), macrophage (MΦ) or natural killer (NK) cells. Animals used were WT, *Neu1* KO, *Neu3* KO, *Neu4* KO, and *Neu3*/*4* DKO mice. The data are presented as cell counts (individual values and mean ± SEM) compared with the WT saline controls (monocytes, 7.4; neutrophils, 4.7; macrophages, 2.3; NK cells, $9.1 \times 10^4$ cells/pouch). Means were compared with those of control groups using one-way ANOVA followed by a Dunnett's *t*-test (*, $p \leq .05$; **, $p \leq .01$; ****, $p \leq .0001$). Raw cell counts are presented in Figure SI1

dermal and muscular layers were determined by microscopy. In the dermal layer of saline-treated *Neu1* KO mice, the levels of leukocytes were increased compared with those in WT mice, but no difference was observed in the muscular layer. In contrast, in LPS-treated *Neu1* KO mice, leukocyte levels were significantly reduced in both dermal and muscular layers. In the latter layer of LPS-treated *Neu3* KO animals, we observed a reduction in leukocytes. *Neu4* KO animals showed increased leukocyte counts in both dermal and muscular layers with LPS treatment, and in muscular layer with saline treatment compared with WT controls. In the *Neu3*/*4* DKO mice, the LPS effect was attenuated and the leukocyte counts were either similar (dermis) or reduced (muscle) compared with those in WT controls, whereas elevated counts in dermis and muscle were observed for saline-treated mice. These observations are consistent with the results of cytometry analyses of serum and pouch exudates (vide supra) and provide additional support to the hypothesis that NEU1 and NEU3 act as positive regulators of infiltration, while NEU4 acts as a negative regulator.

## 3.4 | Effects of neuraminidases on cytokine and chemokine levels in circulation and exudates

Based on the above findings, we concluded that NEU-deficient animals demonstrate altered regulation of leukocyte recruitment in the air pouch model. One potential mechanism for these changes could involve the modulation of cytokine and chemokine production in the animals. To test this, air pouch exudates and serum samples collected at sacrifice from WT, *Neu1* KO and *Neu4* KO mice were analyzed for a panel of 21 different cytokines and chemokines (Table 2, Tables SI3 and SI4).

As expected, levels of pro-inflammatory cytokines and chemokines were undetectable or very low in the exudates of saline-treated WT mice, but were drastically increased in the exudates of LPS-treated animals. The exudates from LPS-treated *Neu1* KO mice showed significantly attenuated levels of a range of inflammatory modulators such as G-CSF, IL-1β, IL-6, IL-10, IL-15, IFN-γ, MIP1-α, MIP1-β, MIP-2, and TNF-α, as well as augmentation of IL-21 (Figures 4 and SI3). Notably, in these mice, the level of IL-33 was increased in saline-treated pouch exudates suggesting basal level induction of this cytokine. The LPS-treated *Neu4 KO* animals had distinct changes in cytokine levels of their exudates. In these mice, we observed attenuation of G-CSF, IFN-γ and MCP-1, and augmentation of GM-CSF, IL-1β, IL-33, MIP1-α, MIP-1β, and MIP-2 in the exudates.

We also examined plasma samples from these animals to observe systemic changes in cytokine and chemokine levels. Similar to exudate samples, the LPS induction of 7 cytokines (G-CSF, RANTES, IL-6, IL-10, MIP1-α, MIP-1β, and MIP-2) was significantly reduced in the plasma samples of *Neu1* KO mice compared with WT mice (Figures 5 and SI3). In contrast, levels of TNF-α were increased in LPS-treated *Neu1* KO mice compared with LPS-treated WT mice. In addition, basal levels of IL-1α, IL-1β, IL-15, IL-25, and MIP-2 were significantly induced in saline-treated *Neu1* KO animals (Figures 5 and SI3). The response of *Neu4* KO mice to LPS was attenuated for 4 cytokines in plasma (G-CSF, RANTES, MIP-1α, and MIP-1β).

To gain insight on the contribution of exudate cells to cytokine levels, we investigated correlations of the cell counts with cytokine levels of the exudates, as well as with those of the plasma. As expected, the cell counts show strong positive correlations with cytokine levels in the exudates as well as with those of the plasma in WT and *Neu4* KO mice (Figure 6A,B). In contrast, in the *Neu1* KO mice, the cell counts show either inverse or no (statistically non-significant) correlation with the majority of the cytokines both in the plasma and in the exudates (Figure 6A,B). In addition, there was no correlation (positive or negative) of the levels of IL-1β, and IL-15 with the other cytokines in the exudates of the *Neu1* KO animals, whereas in the exudates from WT and Neu4 KO mice, both IL-1β and IL-15 positively correlated with other cytokines. Strong positive correlations were, however, observed in the exudates of all mice for IP-10, MCP-1, MIP-1α, MIP-1β, RANTES, and TNF-α.

## 3.5 | Neuraminidase enzymes affect macrophage migration in vitro

The data obtained using the mouse air pouch model clearly indicated that NEU isoenzymes have dramatic

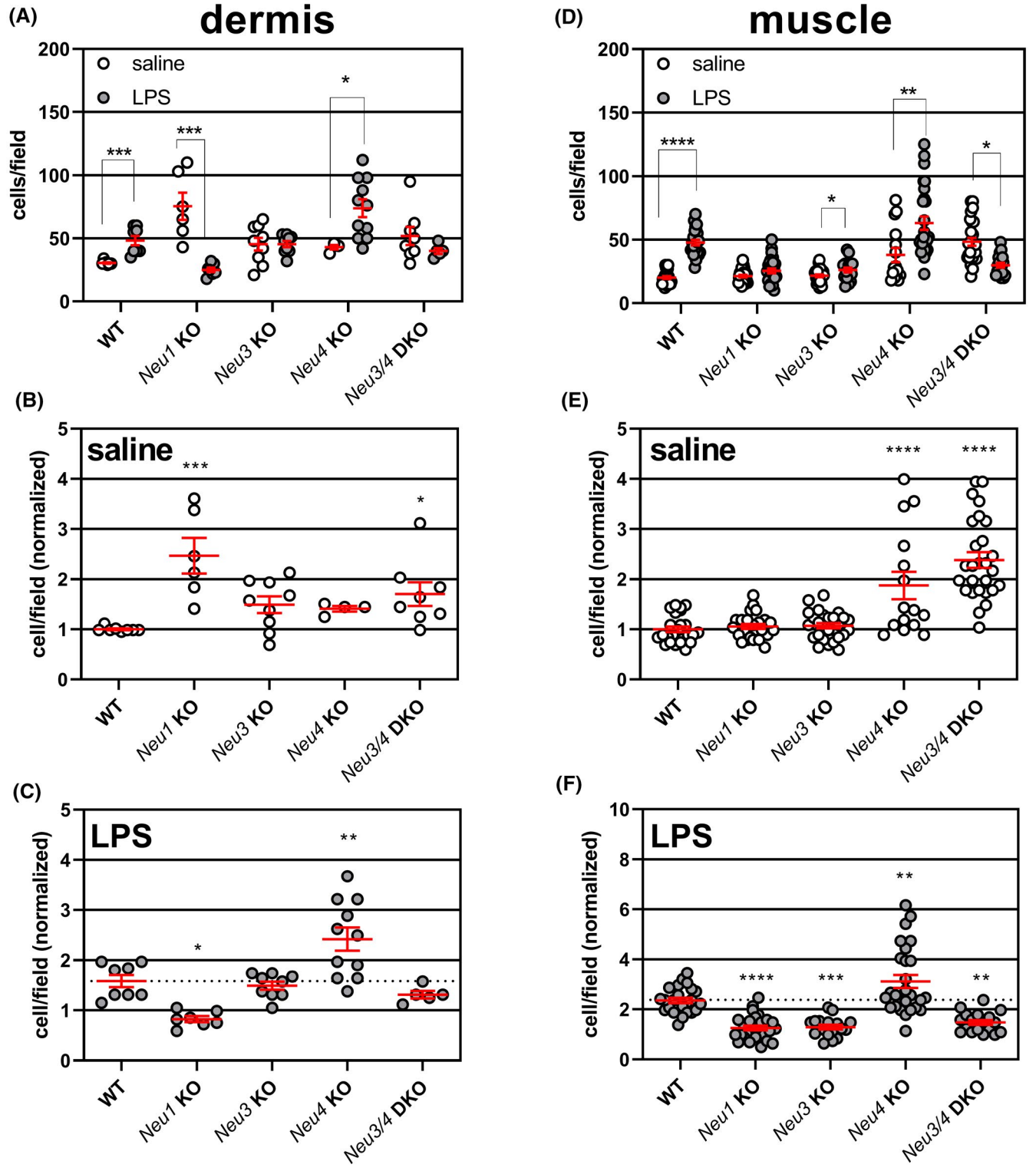



**FIGURE 3** Levels of leukocytes in skin slices from the air pouch model. Tissue samples from the air pouch model were collected, sectioned, and stained with H&E reagent. Regions of tissue were identified as dermis or muscle. See Figure SI2 for representative images of each region in different mice groups. Random fields from each region were used to determine leukocyte counts in saline- (○) or LPS-infused (●) air pouch walls. Panels A and D show raw cell counts for each condition and tissue. Normalized cell counts after saline and LPS treatment are provided for dermis (B and C) and muscle (E and F) layers, respectively. The graphs show individual values and means ± SEM. Conditions were compared using a two-way ANOVA with Holm-Sidak's multiple comparisons (A and D) or one-way ANOVA followed by Dunnett's multiple comparisons (B, C, E, F) (*, $p \leq .05$; **, $p \leq .01$; ***, $p \leq .005$; ****, $p \leq .0001$)

**TABLE 2** Summary of changes to cytokine levels in *Neu1* and *Neu4* KO animals

| Model | | Basal activated[a] | Activated[b] | Attenuated[c] |
|---|---|---|---|---|
| *Neu1* KO | Exudate | IL-33 | IL-21 | G-CSF, IL-1β, IL-6, IL-10, IL-15, IFN-γ, MIP-1α, MIP-1β, MIP-2, TNF-α |
| | Plasma | IL-1α, IL-1β, IL-15, IL-25, MIP-2 | TNF-α | G-CSF, RANTES, IL-6, IL-10, MIP-1α, MIP-1β, MIP-2 |
| *Neu4* KO | Exudate | | GM-CSF, IL-1β, IL-33, MIP-1α, MIP-1β, MIP-2 | G-CSF, IFN-γ, MCP-1 |
| | Plasma | | | G-CSF, RANTES, MIP-1α, MIP-1β |

[a]Basal activated was defined as having a *significant increase in saline for the model relative to saline control.*

[b]Activated was defined as having a *significant positive difference between control LPS and model LPS stimulation.*

[c]Attenuated was defined as having a *significant negative difference between control LPS and model LPS stimulation.*

impact on leukocyte recruitment to the site of inflammation in vivo. To gain further insight into a potential mechanism underlying this phenomenon, we investigated the effect of NEU deficiency on the migration of bone marrow–derived macrophages (BMDMs). For the migration assay, cultured BMDM obtained from the mice with the indicated genotype were seeded into top chamber of a Transwell where the membrane separating the upper and lower chambers had been coated with FN. To the lower chamber of the plate, a chemoattractant (CCL2/MCP-1; 20 ng per well) was added. Nine hours after the addition of BMDM to the upper chamber, we counted leukocytes that had infiltrated the membrane by microscopy. As shown in Figures 7 and SI4, the macrophages from the *Neu1* KO mice were not able to migrate into the FN matrix. In contrast, the macrophages from the *Neu3* KO mice demonstrated increased migration into the matrix. The macrophages from the *Neu4* KO animals showed no difference from WT controls.

## 3.6 | Effect of pharmacological inhibition of NEU on leukocyte subsets

Based on our findings above, we reasoned that pharmacological inhibitors of NEU isoenzymes should be able to recapitulate the effects of gene targeting on cell infiltration in the air pouch model of inflammation. We used previously reported inhibitors[21,23,24] selective for NEU1, NEU3, and NEU4 (Table 3). Each of the inhibitors was dosed at 1 mg/kg body weight and delivered by three IP injections (48 h, 24 h, and 9 h before sacrifice; Table 1 and Figure 8). The dose was as per earlier publications.[21,23,24] We did not observe any significant effects for an inhibitor of NEU1 (**IN1**), which may indicate that the compound was not used at high enough dosage to counteract the most highly expressed NEU isoenzyme. However, we observed an increase in leukocyte counts from exudate after treatment of WT animals with an inhibitor of NEU4 (**IN4**); whereas no significant differences from controls were observed from the inhibitor treatment in animals after LPS stimulation. We noted that the NEU3 inhibitor (**IN3**) reduced the difference in leukocyte counts between saline and LPS treatments (Figure 8A). We examined the effects of the NEU inhibitors on leukocyte subsets (Figures SI5, SI6 and Table SI6). Both IN3 and IN4 treatments resulted in increased MO cell counts for saline treatment relative to control. Furthermore, IN3 treatment gave a significant reduction in MΦ counts after LPS treatment. We note that these experiments were at a single dosage and included a limited number of animals. As a result, follow-up studies will be required to determine the potential of selective NEU inhibitors for affecting leukocyte infiltration.

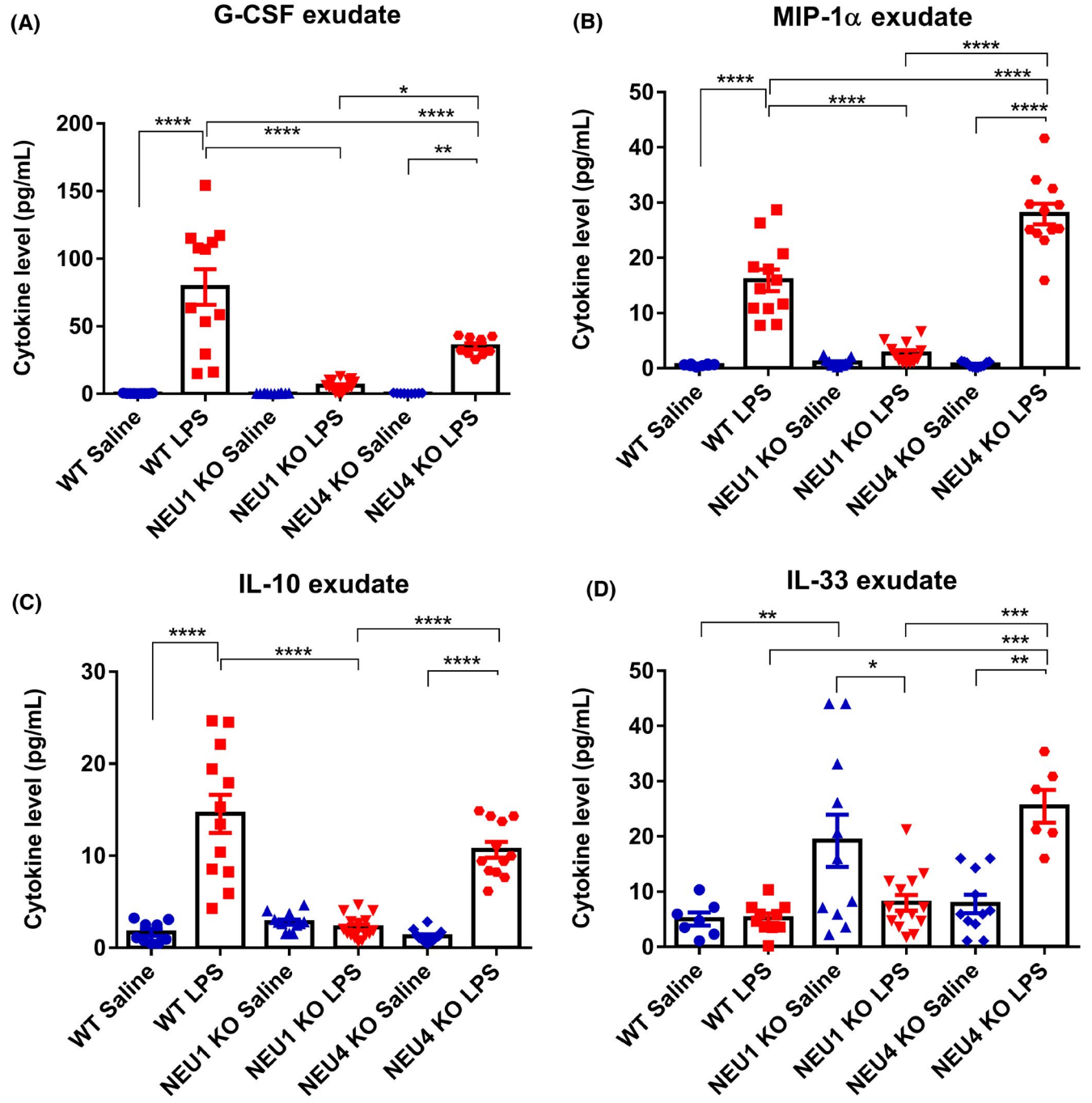



**FIGURE 4** Cytokine and chemokine levels in air pouch exudates. Cytokines were analyzed using the Autoplex Analyser CS1000 system (Perkin Elmer, Waltham, MA) with a commercial ProcartaPlex Mouse Cytokine Panel Assay kit (Thermo Fisher Scientific Inc., Rockford, USA) in accordance with the manufacturer's instructions. (A) G-CSF, (B) MIP-1α, (C) IL-10, and (D) IL-33 are shown as representatives; see Table 2 and Supporting Information for other tested cytokines. Bars represent means ± SEM. Samples were compared with controls using a one-way ANOVA and Tukey's test for post-hoc analysis (*, $p < .05$; **, $p < .01$; ***, $p < .001$; ****, $p < .0001$)

## 4 | DISCUSSION

Using an air pouch model of LPS-induced acute inflammation in gene-targeted mouse strains and a panel of neuraminidase-specific inhibitors, we have investigated the involvement of endogenous neuraminidase enzymes in the migration and recruitment of leukocytes to the site of inflammation.[31] Our data show that NEU1 and NEU3 act as positive regulators of leukocyte recruitment. In contrast, NEU4 acts as a negative regulator of this process. These results were confirmed by IHC of dermal and muscular layers of the air pouch walls. Furthermore, LPS-treated *Neu1* KO mice showed significantly reduced levels of proinflammatory cytokines IL-1β, MIP1-α, MIP-2, and

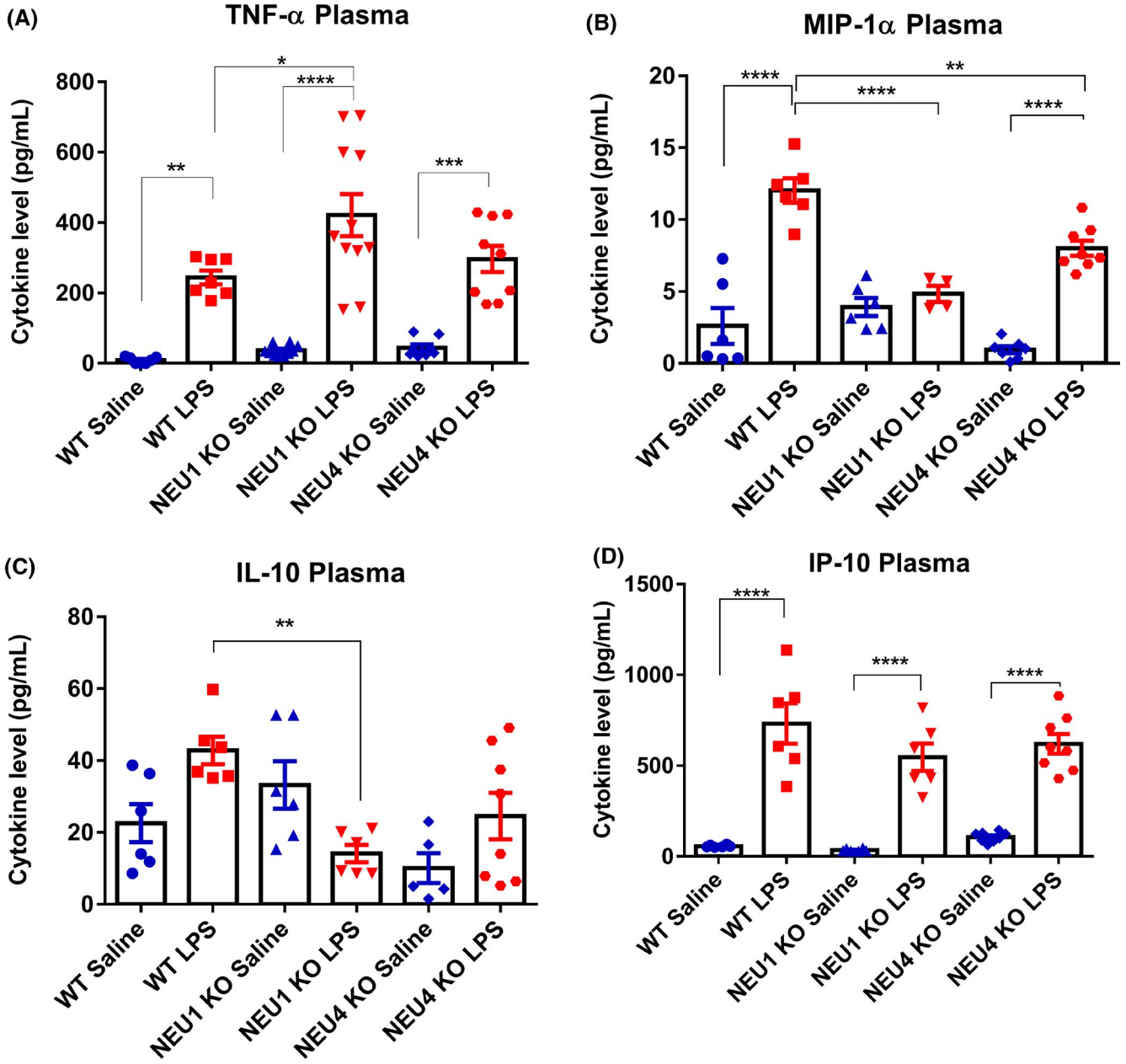



**FIGURE 5** Cytokine and chemokine levels in plasma samples. Cytokines were analyzed as described in the Figure 4 legend. (A) TNF-α, (B) MIP-1α, (C) IL-10, and (D) IP-10 are shown as representatives; see Table 2 and Supporting Information for other tested cytokines. Bars represent means ± SEM. Samples were compared with controls using one-way ANOVA and Tukey's test for post-hoc analysis (*, $p < .05$; **, $p < .01$; ***, $p < .001$; ****, $p < .0001$)

TNF-α in their air pouch exudates as well as an attenuated migratory response of their BMDMs to CCL2 through FN-coated membranes. We conclude from these results that, in general, NEU1 and NEU3 exert pro-inflammatory and NEU4 exerts anti-inflammatory effects.

Our data also demonstrated an increased basal level of inflammation in the control (saline-treated) *Neu1* KO mice with higher numbers of MO and NE in the air pouch exudates, increased leukocyte infiltration in the dermal layers of their pouch walls, and higher concentrations of circulating TNF-α. When interpreting these data, it is important to consider that complete deficiency of NEU1 causes a catabolic block in the degradation of sialylated glycoproteins resulting in their lysosomal storage and leading to systemic metabolic disease, sialidosis.[32] Previous studies in human patients and mouse models of sialidosis have revealed that progressive neuroinflammation and leukocyte infiltration in peripheral tissues are hallmarks of this disease.[33] Signs of neuroinflammation including microastrogliosis and increased brain levels of MIP-1α (CCL3) have been also reported for *Neu3* KO, *Neu4* KO, and *Neu3*/*Neu4* DKO mice, but the levels were substantially

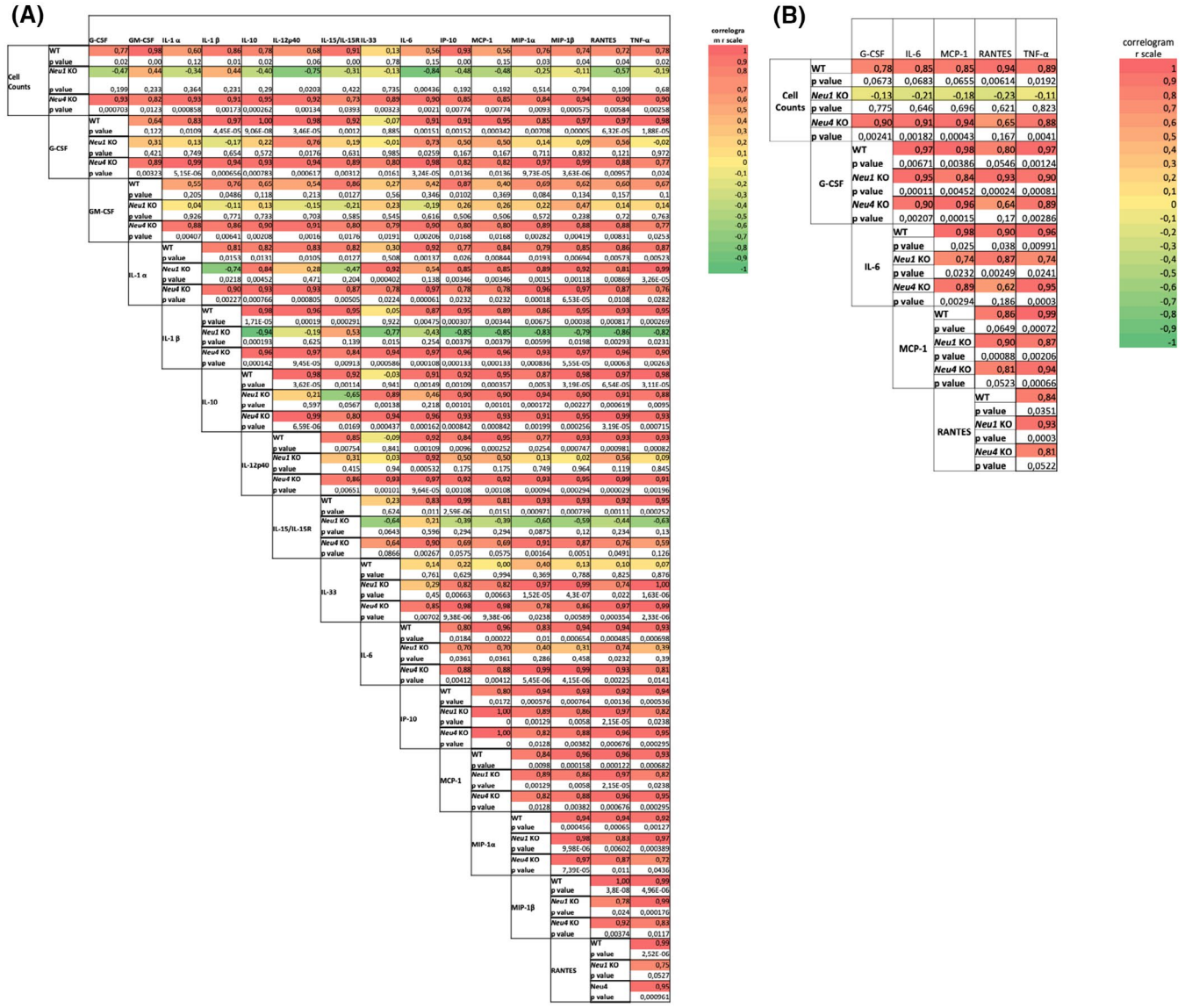



**FIGURE 6** Correlation between exudate cell counts and cytokines. The figures show *r* and *p* values for Pearson correlations between cell counts and cytokine levels in exudates (A) and between exudate cell counts and cytokine levels in plasma (B) of WT, *Neu1* KO, and *Neu4* KO mice with and without treatment with LPS. R values suggesting positive, negative, and no correlations are shown with red, green, and yellow backgrounds, respectively

lower compared with *Neu1* KO mice.[20,34] Thus, increased basal level inflammation in *Neu 1* KO mice is in agreement with previous studies.

Because the results obtained in the *Neu* KO mice suggested involvement of NEU isoenzymes in the regulation of inflammatory response, we tested if specific inhibitors of these enzymes were able to modulate leukocyte recruitment in the air pouch model. The results of our experiments with an inhibitor of NEU1 (**IN1**) were not conclusive due to the high variability between animals. However, treatment with an inhibitor of NEU3 (**IN3**) was consistent with the enzyme's positive regulation of cell infiltration, whereas treatment with an inhibitor of NEU4 (**IN4**) was consistent with negative regulation of leukocyte recruitment. The inhibitors also showed differential effects on leukocyte subsets, which may explain divergence of these results from the gene-targeted animals. We observed that **IN3** treatment reduced leukocyte recruitment in response to LPS in mice in a fashion similar to genetic inactivation of the *Neu3* gene. In contrast **IN4** treatment increased leukocyte recruitment under basal conditions. Interestingly, both the NEU3 and NEU4 inhibitors increased basal levels of MO and NE in the pouch (saline treatment). Although these data are generally supportive of our conclusion that NEU4 is a negative regulator while NEU1 and NEU3 are positive regulators of inflammation, the specific effects are different from those obtained by gene-targeting. It is also important to note that the

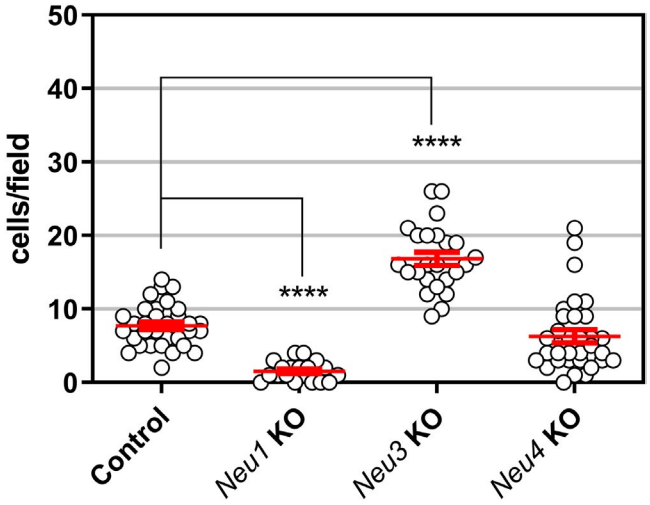
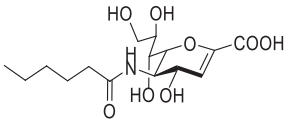
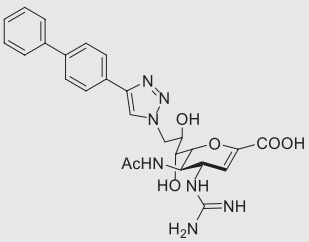
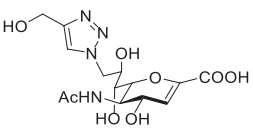



**FIGURE 7** Migration of bone marrow–derived macrophages is regulated by NEU isoenzymes. Monocytes were isolated from the bone marrow of WT, *Neu1* KO, *Neu3* KO, and *Neu4* KO mice. They were differentiated in vitro into macrophages, and $2.5 \times 10^4$ cells were placed in the upper chamber of a Transwell containing CCL2 (20 ng per well) in the lower chamber. Nine hours after the addition of the macrophages to the upper chamber, the number of cells that infiltrated the FN-coated membrane was determined by staining and counting. Cell counts are plotted as means ± SEM. Means were compared using One-way ANOVA followed by Dunnett's *t*-test (****, $p \leq .0001$)

inhibitors were tested at a single dose in a single regimen, and the cohort size was limited. As a result, we cannot exclude that pharmacokinetics, bioavailability, or membrane permeability of the compounds may influence these conclusions. Further studies exploring different doses of the inhibitors will be needed to clarify these effects.

A potential mechanism for the regulation of leukocyte recruitment to the site of inflammation by NEU enzymes is modulation of the production of pro-inflammatory cytokines and chemokines. Indeed, our results indicate that NEU1 activity positively regulates the cytokines that are directly implicated in the recruitment of leukocytes to the site of inflammation such as IL-1β, MIP-1α, and MIP-2.[35–37] NEU1 overexpressing monocytic cells (THP-1) are known to produce increased levels of IL-1β and TNF-α, and conversely, KO of *Neu1* in macrophages results in decreased production of these cytokines.[38] Here, we observed that TNF-α in the plasma was increased in LPS-stimulated *Neu1* KO animals. Furthermore, whereas Sieve et al.[38] observed no effect of *Neu1* KO or of its over-expression on IL-10 production in THP-1 monocytic cells, we observed an inhibitory effect of *Neu1* KO on LPS-induced IL-10 production in the plasma of the mice (Table 2; Figure 5C). It is noteworthy that IL-10 inhibits production of TNF-α.[39] The suppression of IL-10 in response to LPS in *Neu1* KO mice may explain, at least in part, increased TNF-α levels in the plasma in our study. Such an increase in TNF-α was

**TABLE 3** hNEU inhibitors used in this study

| Structure | Identifiers | $IC_{50}$ [μM][d] | | | | Selectivity[e] |
|---|---|---|---|---|---|---|
| | | NEU1 | NEU2 | NEU3 | NEU4 | |
|  | CG14600 **IN1**[a] | 0.42 | 15 | 210 | 440 | NEU1 (36X) |
|  | CG22600 **IN3**[b] | >500 | 6 | 0.58 | 6 | NEU3 (10X) |
|  | CY16600 **IN4**[c] | >500 | >500 | 80 | 0.16 | NEU4 (500X) |

[a]Referred to as compound **11d** in original citation.[24]

[b]Referred to as compound **8b** in original citation.[21]

[c]Referred to as compound **6** in original citation[23]; or C9-(4-hydroxymethyltriazolyl)-DANA (C9-4HMT-DANA); referred to here as CY16600.

[d]$IC_{50}$ values from previous studies against a 4MU-NANA substrate are provided with the indicated citation.

[e]Selectivity based on $IC_{50}$ values cited (and may be an upper limit), with fold-selectivity between the best and next-best target shown in parenthesis.

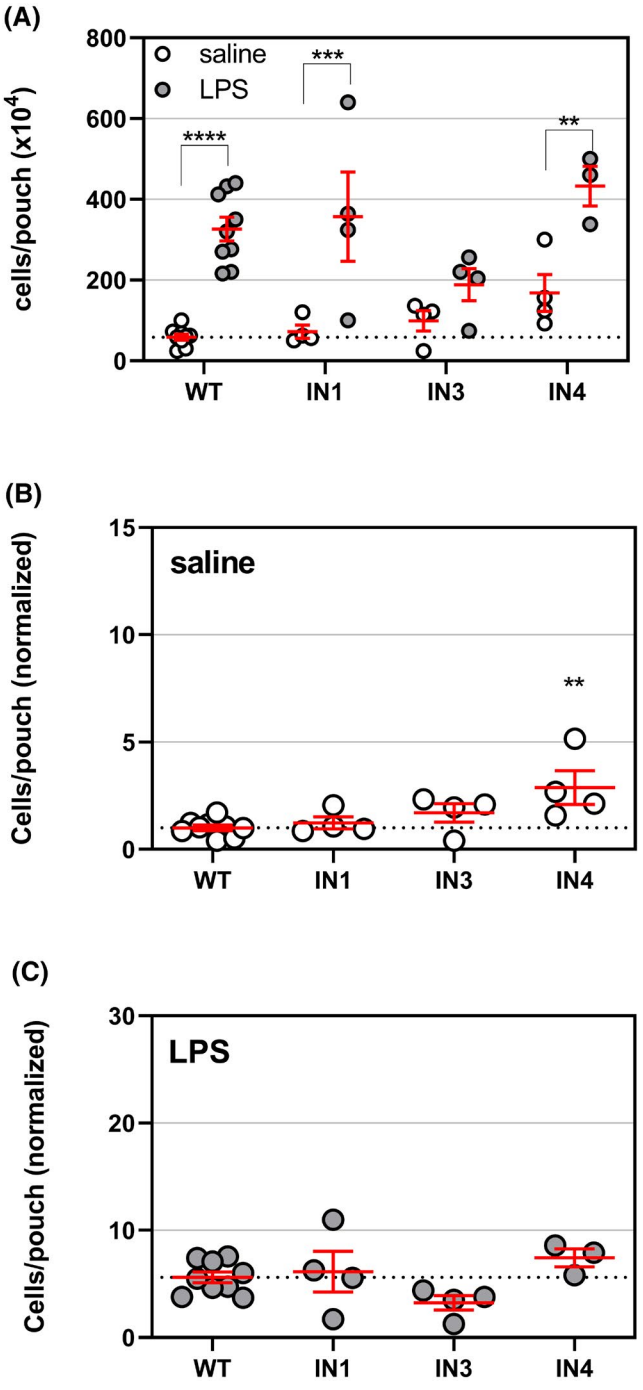



**FIGURE 8** Leukocyte counts in the air pouch model are modulated by NEU inhibitors. WT type mice with air pouches were used in this experiment. The mice were injected with saline (0.90% NaCl; as control) or NEU-specific inhibitors ip (1 mg/kg of body weight; three injections per mouse 48 h, 24 h, and 0 h before infusion of LPS or saline into the air pouches). Nine hours later, the mice were sacrificed and the air pouch exudates were collected, and infiltrated cells were counted by flow cytometry. Inhibitors that target NEU1 (**IN1**, CG14600), NEU3 (**IN3**, CG22600), and NEU4 (**IN4**, CY16600) were used. (A) Total cell counts are presented for saline (○) and LPS (●) treatments. (B) Cell counts for the saline treatment in the *Neu* KO mice are compared with those of WT mice. (C) Cell counts for the LPS treatment are compared with those of WT mice. Data are presented as individual points and mean ± SEM. For panel A, comparisons were made using two-way ANOVA following Bonferroni multiple comparison *t*-test; for panel B & C, comparisons were made using one-way ANOVA with Dunnett's *t*-test for multiple comparison (**, $p \leq .01$; ***, $p \leq .005$; ****, $p \leq .0001$). Raw cell counts for leukocyte subsets are shown in Figure SI5

not observed in the pouch exudate, probably due to the inhibitory effect of *Neu1* KO on leukocyte infiltration. The discordance in the results could be due to different models of *Neu1* KO used in the two studies. Sieve et al.[38] studied *Neu 1* KO effects on cytokines in monocytic cells whereas our investigations were conducted in genetically produced *Neu1* KO mice. Notably, there is significant difference between a knock-down cell line and the organism, as the latter may show differences from a range of cell types rather than from a single cell line. Thus, different experimental approaches and enzyme specificities may account for different results with respect to certain cytokines. The differential effects of NEU isoenzymes on the production of cytokines and chemokines reported here are also supported by earlier studies. For example, Stamatos and coworkers showed that exogenous treatment of purified human MO with neuraminidase from *Clostridium perfringens* increased production of IL-6, MIP-1α (CCL3) and MIP-1β (CCL4), but had no effect on RANTES (CCL5), IL-10, TNF-α, IFN-γ, and IL-1β.[40]

The leukocyte counts in the pouch exudates strongly correlated with cytokine levels in the exudates and in the plasma in WT, and even more in the *Neu4* KO animals, but not in the *Neu1* KO mice (Figure 6). Importantly, strong correlations were observed with a majority of chemokines in the mice of all genetic backgrounds including the NEU1-deficient animals, suggesting that the stress induced by the air pouch and the LPS, was recognized and resulted in chemokine release both locally and systemically. However, the absence of correlation between leukocyte counts and cytokine levels in the *Neu1* KO mice demonstrates that in the absence of NEU1, the cells were not able to react to them and infiltrate the air pouch. Thus, it is tempting to speculate that, in addition to its previously described role in the activation of TLR-4 and FCR-γ immune receptors, NEU1 is also involved in activation of receptors for cytokines.[6,17] In contrast to WT and *Neu4* KO mice, the levels of IL-1β, the major cytokine produced by innate immune cells during the inflammasome induction, are not associated with the other cytokines in the *Neu1* KO. This suggests a specific role of NEU1 in IL-1β production which we intend to investigate in the future.

*Neu4* KO mice, in contrast to *Neu1* KO mice, had fewer changes to cytokine profiles. Despite this, our data regarding cell migration in the air pouch model indicated elevated

accumulation of MO, NE, and NK cells in the *Neu4* KO mice (Figure 2). This observation may be partly explained by elevated cytokine levels observed in exudate samples from these animals. We observed activation of 7 cytokines from *Neu4* KO exudate samples. Among these are cytokines that are known to act as chemoattractants for leukocytes including MO, NE, or NK cells.[35–37,41–43] Together, these data suggest that NEU4 and NEU1 modulate cytokine levels which may explain the changes in leukocyte migration observed in these models. Further experiments will be required to test if cytokine levels are directly or indirectly affected by NEU deficiencies; however, our current data provide evidence for clear differences in cytokine levels between WT, *Neu1* KO and *Neu4* KO mice, and warrant future investigation.

NEU isoenzymes are known to regulate the sialylation levels of cell surface glycoproteins and glycolipids, which may in turn alter the activation threshold of receptors involved in leukocyte migration and/or production of cytokines and chemokines. We previously observed that levels of NEU1 are increased at least 10-fold during the differentiation of MO to MΦ, and that the enzyme is targeted from lysosomes to the cell membrane.[44,45] Furthermore, increased sialylation of cell surface receptors targeted by NEU1, including Fc-γ receptors on MΦ, has been demonstrated in *CathA*$^{S190Aneo}$ mice with ~90% reduction of NEU1 activity.[5,17] The *Neu1* KO mouse is completely deficient in NEU1 and is likely to show enhanced sialylation of NEU1-targeted glycoconjugates including receptors and proteins involved in signaling cascades activated by LPS. In our study, we used LPS, a major component of the cell wall of Gram-negative bacteria, to induce inflammation in the air pouch. The binding of LPS to TLR-4 via CD14 (a GPI-anchored homodimer) induces TLR-4 dimerization and activates a signaling cascade that culminates in the activation of NF-kB, and production of pro-inflammatory cytokines and chemokines.[46] We have previously reported that TLR-4-induced signaling causes activation and translocation of endogenous NEU1 to the cell surface. The activated NEU1 desialylates TLR-4, and this process plays a critical role in the TLR-4-mediated activation of NF-kB. Specifically, in macrophages and dendritic cells, LPS-induced MyD88/TLR4 complex formation was dependent on the removal of α2,3-sialyl residue linked to β-galactoside of TLR4[6,47] Thus *Neu 1* KO mice are likely to have an increased threshold for TLR4-mediated signaling and activation of NF-kB upon stimulation with LPS. This mechanism may explain the reduced production of pro-inflammatory cytokines and chemokines in the *Neu1* KO mouse. Not surprisingly, the neuraminidase potentiates LPS-induced acute lung injury in mice,[48] while siRNA-mediated knock-down of *Neu1* has been found to block LPS induction of TNF-α and IL-1β in human MOs.[38] Besides attenuating TLR-4 signaling, cell surface protein hypersialylation may also affect production of cytokines and chemokines by other mechanisms such as masking of galectin receptors or engagement of inhibitory Siglecs by trans- and cis-acting sialylated epitopes.[49,50] It is noteworthy that LPS/TLR4-induced signaling primes the inflammasome, which upon activation, processes and releases IL-1β.[51] By attenuating TLR-4 signaling, hypersialylation in *Neu1* KO mice also results in reduced production of this cytokine. Further studies are required to clarify specific links between NEU1, LPS-mediated signaling and inflammasome priming/activation.

The rolling and tethering of leukocytes onto endothelial cells is a first step in the extravasation of leukocytes to the sites of inflammation in the body.[31] The sialylated epitopes sLe$^a$ and sLe$^x$ (CD15s) are present on a variety of glycoproteins, such as PSGL-1 and CD44, and are critical for extravasation of normal leukocytes. These sialylated epitopes are also abundantly expressed on cancer cells and mediate their interaction through selectins on endothelial cells. Interestingly, Neu4S, the short cytoplasmic NEU4 isoform expressed in peripheral tissues, can desialylate these binding epitopes.[52] This may suggest a molecular mechanism by which NEU4 could act as a negative regulator of leukocyte recruitment to a site of inflammation.

Our study revealed a positive role for NEU3 in the recruitment of leukocytes. Previous studies have found that neuraminidases can affect leukocyte recruitment by modulating activities of adhesion molecules.[53] Our previous work has suggested that NEU3 mediates desialylation of gangliosides and glycoproteins which may inhibit cis-interactions with β1 integrins (α4β1 and α5β1; also known as VLA4 and VLA5, respectively).[12,54] Here, we confirmed that BMDM from *Neu3* KO animals had increased in vitro chemotaxis through a FN-coated membrane, consistent with this isoenzyme acting as a negative regulator of cell migration in response to a chemoattractant (MCP-1/CCL2). In contrast, *Neu1* KO animals showed reduced migration in vitro. Further studies are required to clarify the mechanism by which NEU1 induces leukocyte transmigration, but it is notable that the MCP-1 receptor, CCR2, is sialylated.[55] BMDM from *Neu4* KO mice did not show differences from control in the migration assay, which may indicate that the enzyme regulates a different aspect of leukocyte recruitment. We hope that our findings will spur future investigations of leukocyte activation to explore the specific mechanisms responsible for these effects more fully.

In conclusion, this study has demonstrated that neuraminidase isoenzymes play a key role in LPS-stimulated acute inflammatory response. These effects were highly dependent upon the NEU isoenzyme tested. We found that NEU1 and NEU3 activity was pro-inflammatory, while

NEU4 activity was anti-inflammatory. Together, these results bolster the case for differential involvement of NEU isoenzymes in the activation and infiltration of leukocytes during inflammation. This discovery could provide new targets for therapeutics and may indicate an unrecognized role for NEU in immune cell regulation. Further work is necessary to determine the specific mechanisms regulated by each enzyme tested here.

## ACKNOWLEDGMENTS

We are grateful for the use of facilities provided by the University of Alberta Department of Chemistry. IHC was performed by Sullen Lamb, Histology Lab Services, University of Alberta.

## DISCLOSURES

MAH, CWC, TG, and AVP are inventors on patent applications related to this work.

## AUTHOR CONTRIBUTIONS

*Designed research*: Md. Amran Howlader, Ekaterina P. Demina, Suzanne Samarani, Ali Ahmad, Alexey V. Pshezhetsky, and Christopher W. Cairo; *Performed research and analyzed data*: Md. Amran Howlader, Ekaterina P. Demina, Suzanne Samarani, and Antoine Caillon; *Contributed new reagents*: Tianlin Guo; *Wrote the manuscript*: Md. Amran Howlader, Ali Ahmad, Alexey V. Pshezhetsky, and Christopher W. Cairo.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the methods and/or supplementary material of this article.

## ORCID

*Md. Amran Howlader* https://orcid.org/0000-0002-1872-3480
*Ekaterina P. Demina* https://orcid.org/0000-0002-0152-6122
*Suzanne Samarani* https://orcid.org/0000-0003-3601-401X
*Tianlin Guo* https://orcid.org/0000-0001-5096-2070
*Antoine Caillon* https://orcid.org/0000-0003-0803-569X
*Ali Ahmad* https://orcid.org/0000-0001-7689-7115
*Alexey V. Pshezhetsky* https://orcid.org/0000-0002-6612-1062
*Christopher W. Cairo* https://orcid.org/0000-0003-3363-8708

## REFERENCES

1. Ley K, Laudanna C, Cybulsky MI, Nourshargh S. Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nat Rev Immunol*. 2007;7:678-689.
2. Rossol M, Heine H, Meusch U, et al. LPS-induced cytokine production in human monocytes and macrophages. *Crit Rev Immunol*. 2011;31:379-446.
3. Miyake K. Innate recognition of lipopolysaccharide by Toll-like receptor 4–MD-2. *Trends Microbiol*. 2004;12:186-192.
4. Feng CG, Stamatos NM, Dragan AI, et al. Sialyl residues modulate LPS-mediated signaling through the Toll-like receptor 4 complex. *PLoS One*. 2012;7:e32359.
5. Pshezhetsky AV, Hinek A. Where catabolism meets signalling: neuraminidase 1 as a modulator of cell receptors. *Glycoconjugate J*. 2011;28:441-452.
6. Amith SR, Jayanth P, Franchuk S, et al. Neu1 desialylation of sialyl α-2,3-linked β-galactosyl residues of TOLL-like receptor 4 is essential for receptor activation and cellular signaling. *Cell Signal*. 2010;22:314-324.
7. Miyagi T, Yamaguchi K. Mammalian sialidases: Physiological and pathological roles in cellular functions. *Glycobiology*. 2012;22:880-896.
8. Feng C, Zhang L, Almulki L, et al. Endogenous PMN sialidase activity exposes activation epitope on CD11b/CD18 which enhances its binding interaction with ICAM-1. *J Leukocyte Biol*. 2011;90:313-321.
9. Gadhoum SZ, Sackstein R. CD15 expression in human myeloid cell differentiation is regulated by sialidase activity. *Nat Chem Biol*. 2008;4:751-757.
10. Zen K, Cui L-B, Zhang C-Y, Liu Y. Critical role of Mac-1 Sialyl Lewis X moieties in regulating neutrophil degranulation and transmigration. *J Mol Biol*. 2007;374:54-63.
11. Quinn MT, Swain SD, Parkos CA, et al. A carbohydrate neoepitope that is up-regulated on human mononuclear leucocytes by neuraminidase treatment or by cellular activation. *Immunology*. 2001;104:185-197.
12. Howlader MA, Li C, Zou C, Chakraberty R, Ebesoh N, Cairo CW. NEU3 is a negative regulator of LFA-1 adhesion. *Front Chem*. 2019;7:791.
13. Cross AS, Sakarya S, Rifat S, et al. Recruitment of murine neutrophils in vivothrough endogenous sialidase activity. *J Biol Chem*. 2003;278:4112-4120.
14. Sakarya S, Rifat S, Zhou J, et al. Mobilization of neutrophil sialidase activity desialylates the pulmonary vascular endothelial surface and increases resting neutrophil adhesion to and migration across the endothelium. *Glycobiology*. 2004;14:481-494.
15. Nan X, Carubelli I, Stamatos NM. Sialidase expression in activated human T lymphocytes influences production of IFN-gamma. *J Leukoc Biol*. 2007;81:284-296.
16. Stamatos NM, Carubelli I, van de Vlekkert D, et al. LPS-induced cytokine production in human dendritic cells is regulated by sialidase activity. *J Leukoc Biol*. 2010;88:1227-1239.
17. Seyrantepe V, Iannello A, Liang F, et al. Regulation of phagocytosis in macrophages by neuraminidase 1. *J Biol Chem*. 2010;285:206-215.
18. Sin YM, Sedgwick AD, Chea EP, Willoughby DA. Mast cells in newly formed lining tissue during acute inflammation: a six day air pouch model in the mouse. *Ann Rheum Dis*. 1986;45:873-877.
19. Smutova V, Albohy A, Pan X, et al. Structural basis for substrate specificity of mammalian neuraminidases. *PLoS One*. 2014;9:e106320.
20. Pan X, De Aragao CBP, Velasco-Martin JP, et al. Neuraminidases 3 and 4 regulate neuronal function by catabolizing brain gangliosides. *FASEB J*. 2017;31:3467-3483.

21. Guo T, Datwyler P, Demina E, et al. Selective inhibitors of human neuraminidase 3. *J Med Chem*. 2018;61:1990-2008.
22. Zhang Y, Albohy A, Zou Y, Smutova V, Pshezhetsky AV, Cairo CW. Identification of selective inhibitors for human neuraminidase isoenzymes using C4, C7-modified 2-deoxy-2,3-didehyd ro-N-acetylneuraminic acid (DANA) analogues. *J Med Chem*. 2013;56:2948-2958.
23. Albohy A, Zhang Y, Smutova V, Pshezhetsky AV, Cairo CW. Identification of selective nanomolar inhibitors of the human neuraminidase, NEU4. *ACS Med Chem Lett*. 2013;4:532-537.
24. Guo T, Heon-Roberts R, Zou C, Zheng R, Pshezhetsky AV, Cairo CW. Selective inhibitors of human neuraminidase 1 (NEU1). *J Med Chem*. 2018;61:11261-11279.
25. Lindberg B, Lindh F, Lönngren J, Lindberg AA, Svenson SB. Structural studies of the O-specific side-chain of the lipopolysaccharide from Escherichia coli O 55. *Carbohydr Res*. 1981;97:105-112.
26. Cronstein BN, Naime D, Ostad E. The antiinflammatory mechanism of methotrexate. Increased adenosine release at inflamed sites diminishes leukocyte accumulation in an in vivo model of inflammation. *J Clin Investig*. 1993;92:2675-2682.
27. Tessier PA, Naccache PH, Clark-Lewis I, Gladue RP, Neote KS, McColl SR. Chemokine networks in vivo: involvement of C-X-C and C-C chemokines in neutrophil extravasation in vivo in response to TNF-alpha. *J Immunol*. 1997;159:3595-3602.
28. Terkeltaub R, Baird S, Sears P, Santiago R, Boisvert W. The murine homolog of the interleukin-8 receptor CXCR-2 is essential for the occurrence of neutrophilic inflammation in the air pouch model of acute urate crystal-induced gouty synovitis. *Arthritis Rheum*. 1998;41:900-909.
29. Nguyen KD, Fentress SJ, Qiu Y, Yun K, Cox JS, Chawla A. Circadian Gene *Bmal1* regulates diurnal oscillations of Ly6C$^{hi}$ inflammatory monocytes. *Science*. 2013;341:1483-1488.
30. Senger DR, Perruzzi CA, Streit M, Koteliansky VE, de Fougerolles AR, Detmar M. The α1β1 and α2β1 integrins provide critical support for vascular endothelial growth factor signaling, endothelial cell migration, and tumor angiogenesis. *Am J Pathol*. 2002;160:195-204.
31. Muller WA. Getting leukocytes to the site of inflammation. *Vet Pathol*. 2013;50:7-22.
32. Pshezhetsky AV, Ashmarina M. Lysosomal multienzyme complex: biochemistry, genetics, and molecular pathophysiology. *Prog Nucleic Acid Res Mol Biol*. 2001;69:81-114.
33. Wu X, Steigelman KA, Bonten E, et al. Vacuolization and alterations of lysosomal membrane proteins in cochlear marginal cells contribute to hearing loss in neuraminidase 1-deficient mice. *Biochim Biophys Acta*. 2010;1802:259-268.
34. Kho I, Pan X, Cairo C, Morales CM, Pshezhetsky A. Study of a novel neuraminidase 1 knockout mouse links the pathology of sialidosis in the nervous, renal and reproductive system. *Mol Genet Metab*. 2020;129:S88.
35. Sherry B, Espinoza M, Manogue KR, Cerami A. Induction of the chemokine β peptides, MIP-1 α and MIP-1 β, by lipopolysaccharide is differentially regulated by immunomodulatory cytokines γ-IFN, IL-10, IL-4, and TGF-β. *Mol Med*. 1998;4:648-657.
36. Diab A, Abdalla H, Li HL, et al. Neutralization of macrophage inflammatory protein 2 (MIP-2) and MIP-1α attenuates neutrophil recruitment in the central nervous system during experimental bacterial meningitis. *Infect Immun*. 1999;67:2590-2601.
37. Lotfi N, Thome R, Rezaei N, et al. Roles of GM-CSF in the pathogenesis of autoimmune diseases: an update. *Front Immunol*. 2019;10:1265.
38. Sieve I, Ricke-Hoch M, Kasten M, et al. A positive feedback loop between IL-1β, LPS and NEU1 may promote atherosclerosis by enhancing a pro-inflammatory state in monocytes and macrophages. *Vasc Pharmacol*. 2018;103:16-28.
39. Armstrong L, Jordan N, Millar A. Interleukin 10 (IL-10) regulation of tumour necrosis factor alpha (TNF-alpha) from human alveolar macrophages and peripheral blood monocytes. *Thorax*. 1996;51:143-149.
40. Stamatos NM, Curreli S, Zella D, Cross AS. Desialylation of glycoconjugates on the surface of monocytes activates the extracellular signal-related kinases ERK 1/2 and results in enhanced production of specific cytokines. *J Leukocyte Biol*. 2004;75:307-313.
41. Pelletier M, Bouchard A, Girard D. In vivo and in vitro roles of IL-21 in inflammation. *J Immunol*. 2004;173:7521-7530.
42. Choi Y-S, Choi H-J, Min J-K, et al. Interleukin-33 induces angiogenesis and vascular permeability through ST2/TRAF6-mediated endothelial nitric oxide production. *Blood*. 2009;114:3117-3126.
43. Gomez-Cambronero J, Horn J, Paul CC, Baumann MA. Granulocyte-macrophage colony-stimulating factor is a chemoattractant cytokine for human neutrophils: involvement of the ribosomal p70 S6 kinase signaling pathway. *J Immunol*. 2003;171:6846-6855.
44. Liang F, Seyrantepe V, Landry K, et al. Monocyte differentiation up-regulates the expression of the lysosomal sialidase, Neu1, and triggers its targeting to the plasma membrane via major histocompatibility complex class II-positive compartments. *J Biol Chem*. 2006;281:27526-27538.
45. Stamatos NM, Liang F, Nan X, et al. Differential expression of endogenous sialidases of human monocytes during cellular differentiation into macrophages. *FEBS J*. 2005;272:2545-2556.
46. Kawasaki T, Kawai T. Toll-like receptor signaling pathways. *Front Immunol*. 2014;5:461.
47. Pshezhetsky AV, Ashmarina LI. Desialylation of surface receptors as a new dimension in cell signaling. *Biochemistry (Moscow)*. 2013;78:736-745.
48. Feng C, Zhang L, Nguyen C, et al. Neuraminidase reprograms lung tissue and potentiates lipopolysaccharide-induced acute lung injury in mice. *J Immunol*. 2013;191:4828-4837.
49. Schauer R. Sialic acids as regulators of molecular and cellular interactions. *Curr Opin Struct Biol*. 2009;19:507-514.
50. Pearce OMT, Läubli H. Sialic acids in cancer biology and immunity. *Glycobiology*. 2016;26:111-128.
51. Sutterwala FS, Haasken S, Cassel SL. Mechanism of NLRP3 inflammasome activation. *Ann N Y Acad Sci*. 2014;1319:82.
52. Shiozaki K, Yamaguchi K, Takahashi K, Moriya S, Miyagi T. Regulation of sialyl lewis antigen expression in colon cancer cells by sialidase NEU4. *J Biol Chem*. 2011;286:21052-21061.
53. Wright RD, Cooper D. Glycobiology of leukocyte trafficking in inflammation. *Glycobiology*. 2014;24:1242-1251.
54. Jia F, Howlader MA, Cairo CW. Integrin-mediated cell migration is blocked by inhibitors of human neuraminidase. *Biochim Biophys Acta*. 2016;1861:1170-1179.

55. Matsubara K, Matsushita Y, Sakai K, et al. Secreted ectodomain of sialic acid-binding Ig-like lectin-9 and monocyte chemoattractant protein-1 promote recovery after rat spinal cord injury by altering macrophage polarity. *J Neurosci*. 2015;35:2452-2464.

## SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

**How to cite this article:** Howlader MA, Demina EP, Samarani S, et al. The Janus-like role of neuraminidase isoenzymes in inflammation. *FASEB J*. 2022;36:e22285. doi:10.1096/fj.202101218R