Modulation of leukocyte adhesion and cell migration by human neuraminidase enzymes

by

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Abstract

The human neuraminidases (NEU1, NEU2, NEU3, and NEU4) are a family of enzymes implicated in pathologies including cancer and diabetes. Our group has developed selective inhibitors for these enzymes that have been employed as tools to understand their biological roles. Several reports have linked neuraminidase activity to the regulation of cell migration in cancerous and normal cells, as well as to the function of leukocytes. This thesis has investigated the role of human neuraminidase enzymes (hNEU) in cell migration and adhesion.

In Chapter 2, we used an *in vitro* cell migration assay on fibronectin (FN) coated surfaces to investigate the role of these enzymes in integrin-mediated cell migration. We observed that neuraminidase inhibition caused significant retardation of cell migration in breast cancer (MDA-MB-231) and prostate cancer (PC-3) cell lines when treated with inhibitors of NEU3 and NEU4. In contrast, inhibition of NEU1 caused a significant increase in cell migration for these same cell lines. Both NEU1 and NEU3 inhibition caused significant reduction of β1 integrin endocytosis in both PC-3 and MDA-MB-231 cell lines. We concluded that blockade of human neuraminidase enzymes with isoenzyme-selective inhibitors has significant potential in the development of anti-cancer or wound healing therapeutics.

In Chapter 3, we investigated the influence of hNEU on the adhesion of leukocytes *in vitro*. Within the plasma membrane, glycoconjugate-receptor interactions play an important role in the regulation of cell-cell interactions. We investigated the mechanism and activity of the hNEU isoenzyme, NEU3, on T cell

adhesion receptors. The enzyme is known to prefer glycolipid substrates and we confirmed that exogenous enzyme altered the glycolipid composition of cells. Enzymatic activity of NEU3 resulted in re-organization of LFA-1 into large clusters on the membrane. This change was facilitated by an increase in the lateral mobility of LFA-1 upon NEU3 treatment. Changes to the lateral mobility of LFA-1 were specific for NEU3 activity, and we observed no significant change in diffusion when cells were treated with a bacterial NEU (NanI). Furthermore, we found that NEU3 treatment of cells increased surface expression levels of LFA-1. We observed that NEU3-treated cells had suppressed LFA-1 adhesion to an ICAM-1 coated surface using an *in vitro* static adhesion assay. These results suggest that modulation of glycoconjugate composition contributes to the regulation of integrin activity. Additionally, NEU3 may be implicated as a negative regulator of the inflammatory cascade through its regulation of LFA-1.

In Chapter 4, we investigated the role of NEU isoenzymes in acute inflammation in mice with an *in vivo* air pouch model. We observed that mice showed significantly reduced leukocyte recruitment in animals with reduced NEU1 or NEU3 enzyme activity. In contrast, knocking out the NEU4 isoenzyme (NEU4 KO) or NEU4 in combination with NEU3 (NEU3/4 DKO), showed significantly increased leukocyte recruitment. These effects were quantified on leukocyte subpopulations including monocytes, neutrophils, macrophages, and NK cells in isoenzyme selective neuraminidase KO mice. Changes in NEU expression corresponded to changes in systemic cytokine levels, with reduced expression of G-CSF, IL-6, TNF-γ, IP-10, and RANTES in NEU1 KO mice treated with LPS.

LPS treated NEU4 KO mice had reduced expression of G-CSF, TNF-ϒ, and IP-10, and increased expression of IL-21. Immunohistochemistry (IHC) of tissue collected from the air pouch model confirmed changes to leukocyte recruitment. Using isoenzyme-selective inhibitors designed in our laboratory, we tested the hNEU enzymes as anti-inflammatory targets. A selective NEU4 inhibitor increased leukocyte recruitment and could act as an immune stimulant. The results show human neuraminidase enzymes are a modulator of acute inflammation.

Preface

Chapter 2 is a collaborative work among members of the Cairo group, (UofA). Caishun Li performed SPT, imaging, homotypic aggregation, and HPTLC experiments; Radhika Chakraberty performed LC-MS analysis of glycolipids; Chunxia Zou performed in vitro adhesion assays. Md. Amran Howlader conducted Western blot, endocytosis, flow cytometry, and lectin blot experiments. A version of this chapter has been submitted for publication with Md. Amran Howlader as the first author.

Chapter 3 is also a collaborative work among members of the Cairo group (U of A). Compounds used in these studies were synthesized and characterized by Yi Zhang and Tianlin Guo; NEU1-HEK cells were provided by Alexey V. Pshezhetsky (University de Montreal). Md. Amran Howlader conducted migration, western blot, lectin blot, and viability studies. A version of this manuscript has been submitted for publication with Md. Amran Howlader as the first author.

Chapter 4 is a collaborative work between the Cairo group (UofA), Pshezhetsky Group (University de Montreal), and Dr. Ali Ahmed (University de Montreal). Mouse experiments were approved by the Centre de Recherche/Chu Sainte-Justine (protocol #710). Compounds used in these studies were synthesized and characterized by Tianlin Guo (UofA); *in vivo* experiments were carried out in the lab of Prof. Alexey V. Pshezhetsky (University de Montreal) by Md. Amran Howlader and Ekatrina Demina. FACS analysis of leukocyte subsets was carried out by Md. Amran Howlader with help from Suzzane Summarani and Prof. Ali Ahmed. Slide preparation for IHC was performed by Sullen Lamb, Histology Lab Services (UofA). Some authors (MAH, TG, CWC, AVP) are inventors on a provisional patent filed related to this work. Md. Amran Howlader wrote the first draft of a manuscript describing this work, which is in preparation for publication.

Dedication

To my departed father who gave me everything so I can learn,
To my mother for making me who I am today
To my wife, love of my life, my anchor Farzana Yesmin
To my parents-in-law, for trusting in me
To Farnaaz, and Azvinna, my constrant inspirations
and
To the loving memory of my grand parents
Moslem Ali Sarder (1940-2019) and Fazilatun Nessa (1951-2016)

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I want to take this opportunity to express my gratitude to all the great people that I had the chance to work with in the last six years, whose cooperation made this thesis see the end of the road. I would like to thank my supervisor, my mentor, and more, Dr. Christopher Cairo for his support, nourishment, teachings, and guidance. He taught me the way science works in North America, the way to think about problems, the process of research management, writing grants & applications, and especially the way to express scientific findings.

I also want to thank Dr. Alexey V. Pshezhetsky (University de Montreal) for his support in conducting the *in vivo* studies described in Chapter 5. I would like to thank Dr. Ekatrina Demina for her hands-on support in conducting *in vivo* experiments and follow-up studies; and Dr. Suzzane Sumarani for conducting the FACS analysis.

I will forever be obliged to Dr. Carson Li, for his hands-on teaching of microscopy and cell culture; Cecilia Zou for her teaching of enzyme production, protein purification, and western blots; and Dr. Tianlin Guo for agreeing to all my demands for chemical synthesis of selective neuraminidase inhibitors used throughout this thesis. Thanks to all the past and present members of the Cairo group for their continuous support and collaboration.

I would like to express my gratitude to agencies that provided support of my work through grants and scholarships including: AITF, NSERC, GlycoNet, and the University of Alberta.

I want to acknowledge my parents, who groomed me and willed me to pursue science. My mother, Shamima Sultana, who made me the person I am today. Thanks to my siblings Sumia Sultana and Zishan Zulfiqur for their support and sacrifices. Finally, I must admit that pursuing a Ph.D. degree would have been an impossible task for me without the constant support and countless sacrifices given by my wife Farzana Yesmin. I am forever grateful to you, almighty Allah, for giving me the strength to finish this work.

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List of abbreviations

4MU-NANA	2′-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid
ALI	Alveolar lung injury
CathA	Cathepsin A
CD	Cluster of differentiation
CDF	Cumulative distribution function
CTB	Cholera toxin subunit B
DANA	2-deoxy-2,3-didehydro-N5-acetylneuraminic acid
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptor
FN	Fibronectin
GAGs	Glycosaminoglycans
GAL	β-galactosidase
Gal	Galactose
G-CSF	Granulocyte colony-stimulating factor
GH	Glycosyl hydrolase
Glc	Glucose
GSL	Glycosphingolipid
GST	Glutathione S-transferase
GT	Glycosyl transferase
GTs	Glycosyltransferases
HA	Hemagglutinin

hNEU	Human neuraminidase enzyme
HexA	Hexosaminidase A
GCase	Galactosidase glucocerebrosidease
HPLC	High-performance liquid chromatography
HPTLC	High-performance thin layer chromatography
ICAM-1	Inter-cellular adhesion molecule-1
IL-4	Interleukin-4
LacCer	Lactosyl ceramide
LAD I	Leukocyte adhesion deficiency type I
LDL	Low-density lipoprotein
LFA-1	Leukocyte functional antigen-1
MAA	Maackia amurensis agglutinin
NanI	Neuraminidase from C. perfringens
NEU	Neuraminidase (Human)
NFκB	Nuclear factor-κB
PBMC	Peripheral blood mononuclear cells
PMA	Phorbol 12-myristate 13-acetate
PNA	Peanut agglutinin
PrpSc	Prion protein
SGC	Sialoglycoconjugates
SNA	Sambucus nigra agglutinin
SPT	Single-particle tracking

STs	Sialyltransferases
TLC	Thin layer chromatography
TLRs	Toll-like receptors
TNF	Tumor necrosis factor
VLA-4	Very-late antigen-4

1. Introduction

1.1 **Sialic acids coat the outside of mammalian cells**

The fluid mosaic model proposes the plasma membrane is a lipid bilayer structure teeming with phospholipids, in which proteins are afloat.[1],[2] Minor but important components of the membrane include glycolipids, cholesterol, glycoproteins, and lipid rafts. The total composition of glycolipids in the membrane is typically less than 5%, but they are essential to cellular functions.[3],[4]

Cells are the building blocks of life, and all living cells are coated with sugars. The first examples to recognize this aspect of cell biology were published by Chambers in the 1940s when they detected organic material coated in layers of some invertebrate eggs[5] and protozoa[6] (1960). In 1962, Gasic et al. was the first to provide histochemical evidence that mammalian cells were abundantly coated with carbohydrates.[7] Subsequent experiments by Rambourg and Leblond investigated 50 different cell types from rat by staining with periodic acid-Schiff and iron-Prussian blue techniques (detecting glycoproteins and carbohydrates respectively) concluded that this carbohydrate-rich cell coating is common in all vertebrate cells (Figure 1.1).[8]

Indeed, the extracellular coating of cells by oligosaccharides is now known to be a universal feature of all kingdoms of life.[9] This important cellular structure has been dubbed the *glycocalyx*, and serves critical structural and functional roles in diverse organisms.

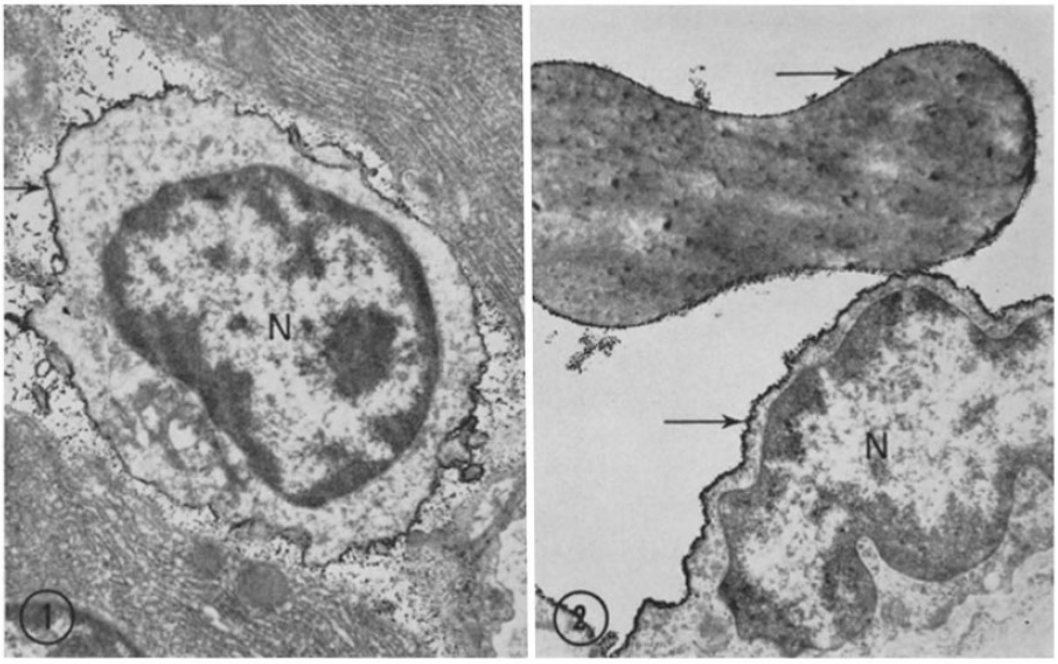


Figure 1.1. Electron microscope images of tissues stained to reveal glycocalyx. (1) Lymphocytes: heavy dark line represents the accumulation of thorium particles on the surface of the lymphocytes. (2) Red blood cells: A layer of colloidal particles marked by the arrows indicate the presence of carbohydrate-rich coat. Images from Rambourg and Leblond, *J Cell Biology*, 1967; reproduced with permission from the publisher.[8]

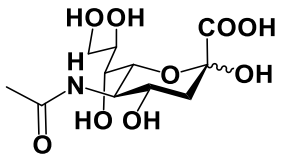
1.1.1 The Glycocalyx - A cellular interface

After the discovery of the glycocalyx, much work was undertaken to understand the constituents of its structure. The glycocalyx consists of a polymeric network of proteoglycans, glycosaminoglycan (GAGs), glycoproteins, and adherent plasma glycoproteins. Luft provided the first electron microscope evidence of the glycocalyx as a small irregular-shaped layer of approximately 50-100 nm in 1966.[10] Later, other experiments suggested that the glycocalyx layer can be as thick as 0.5 μm.[11,12]

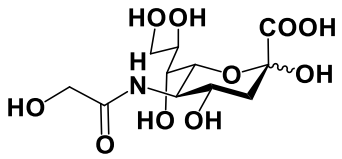
The presence of *N*-acetyl neuraminic acid (Neu5Ac, also called sialic acid) was first detected by Gasic et al., who in 1962 reported ascites tumors contained a Hale-positive coat, which was later identified as neuraminic acid-containing glycans.[7,13] This result first identified neuraminic acid as an integral part of the glycocalyx structure. The glycocalyx structure can be destroyed by treatment with hyaluronidase.[11] Furthermore, glycans of the glycocalyx are directly or indirectly linked with proteins, as some proteases destroy the coat as evident from reduction of the Hale-positive signal.[13] This transformation indicates that enzymes can be involved in restructuring the glycocalyx, and these interactions may be biologically relevant.

1.1.2 Neuraminic acids

Neuraminic acids, also called sialic acids, are a family of more than 50 different sugars with a common characteristic of being 9-carbon α-keto acids. Blix first isolated Neu5Ac in 1936 from bovine submaxillary mucin[14] and later named it as sialic acid.[15] The most common sialic acid found in vertebrates is 5-*N*-acetylneuraminic acid [5-acetamido-2-keto-3,5-dideoxy-D-glycero-D-galactononononic acid] (Neu5Ac, **1.1**). Sialic acid is found in animals, protozoa, bacteria, fungi, and viruses; however, there is still no clear evidence of sialic acid found in plants.[16,17]



Neu5Ac (1.1)



Neu5Gc (1.2)

Figure 1.2: Structure of Neu5Ac and NEU5Gc

In experiments with Sarcoma 37 cells, Dr. Weiss discovered that removal of neuraminic acid from cells using neuraminidase (NEU), an enzyme that cleaves neuraminic acid, made the cells deformed.[18] Subsequent experiments from Kemp in 1968 showed neuraminic acid had effects on cell aggregation.[19] This indicated that neuraminic acids have marked effects on cell function. While most animals produce Neu5Gc (1.2 in **Figure 1.2**), rather than Neu5Ac, due to the deletion of the CAMH gene (a gene that responsible for the synthesis of NEU5Gc) in humans,[20] only Neu5Ac is natually produced in humans. However, Neu5Gc can be found in humans as a contaminant, which is likely due to dietary consumption.[21]

Neuraminic acid is most commonly bound to Asn residues (N-linked) or Ser/Thr residues (O-linked) linked glycoprotein structures in mammals.[22] The structure of N-linked glycans often contain neuraminic acid in NeuAc-α(2–3)-Gal-β(1–4)-GlcNAc or NeuAc-α(2–6)-Gal-β(1–4)-GlcNAc linkages.[23] The O-linked glycans are often found in mucins and contain neuraminic acid attached via GalNAc-Ser/Thr linkage.[24] Figure **1.3** shows some generalized glycan structures found in human cells using the Consortium for Functional Glycomics (CFG) symbol nomenclature.[25]

There are two exceptional characteristics associated with neuraminic / sialic acids. The first is the negative charge carried by the C1 carboxylate at physiological pH. Secondly, they tend to be present as the terminal sugars of glycoconjugates.[23] Furthermore, neuraminic acids can contain modifications such as acetylation, methylation, and phosphorylation.[23] These modifications of neuraminic acids can alter their physiological function. For example, influenza C virus that causes flu has been shown to bind only to 9-*O*-acetylated Neu5Ac.[26] The glycosidic linkage between neuraminic acid and the rest of the glycan structure also plays a significant role in its recognition. Neuraminic acids are an important component of glycoconjugates that influence inter-molecular interactions[16] and biological function.[27]

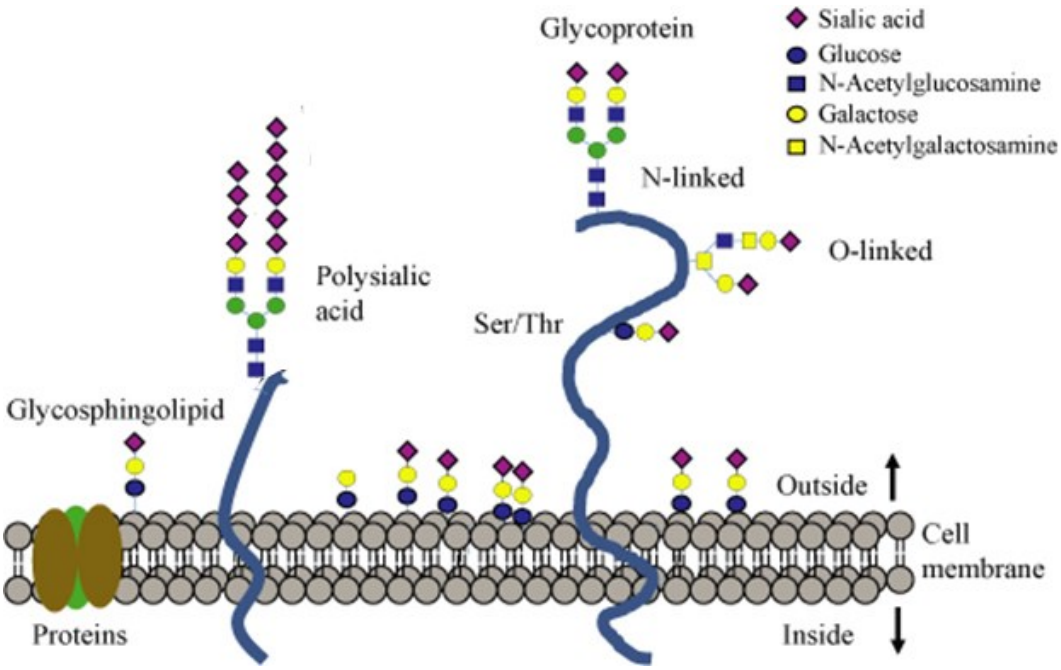


Figure 1.3. Sialic acid-containing glycoconjugates at the plasma membrane. Cells are coated with sialic acids, these may be N-linked or O-linked to proteins, or else attached to ceramide to form glycolipids. This figure was adapted from Varki et. al.[27] CFG symbol nomenclature is used to represent individual carbohydrate residues.[25]

1.1.3 Glycosphingolipids (GSLs)

Glycoproteins and glycolipids, known collectively as *glycoconjugates*, are important components of cells and are responsible for a diverse range of cellular functions. An extensive review of glycan function is beyond the scope of this work;[27] however, a brief summary of the structure and role of glycosphingolipids (GSLs) will provide important context for this thesis.

GSLs are the primary glycolipids found in the membranes of mammalian cells. Structurally, they consist of a glucosyl-ceramide (Glc-Cer) moiety, which is elaborated with additional carbohydrate residues.[28,29,30] Gangliosides are a subclass of GSLs that contain sialic acids within the glycan.[31] Examples of GSL structures are shown in **Figs 1.4** and **1.5**.

Klenk et al. were first to report gangliosides in 1938.[32] Gangliosides are most abundant in neuronal cells, but are also found in different cell types in lower quantity.[33,34] Due to the variability of the glycoconjugate structures, a convention was introduced by Svennerholm in 1994[35] which abbreviates the structure of individual GSLs according to their order of chromatographic separation. For example, GM1 is a ganglioside (hence the "G") containing one sialic acid (mono-M, di-D, tri-T) and migrates the least on thin layer chromatography (TLC), which is represented by the number 1. Similarly, GM2 and GM3 are other mono-sialo gangliosides with a different chromatographic retention. Figure **1.4** shows the structures of the four most abundant gangliosides found in the adult mammalian brain (GM1, GD1a, GD1b, and GT1b).[23]

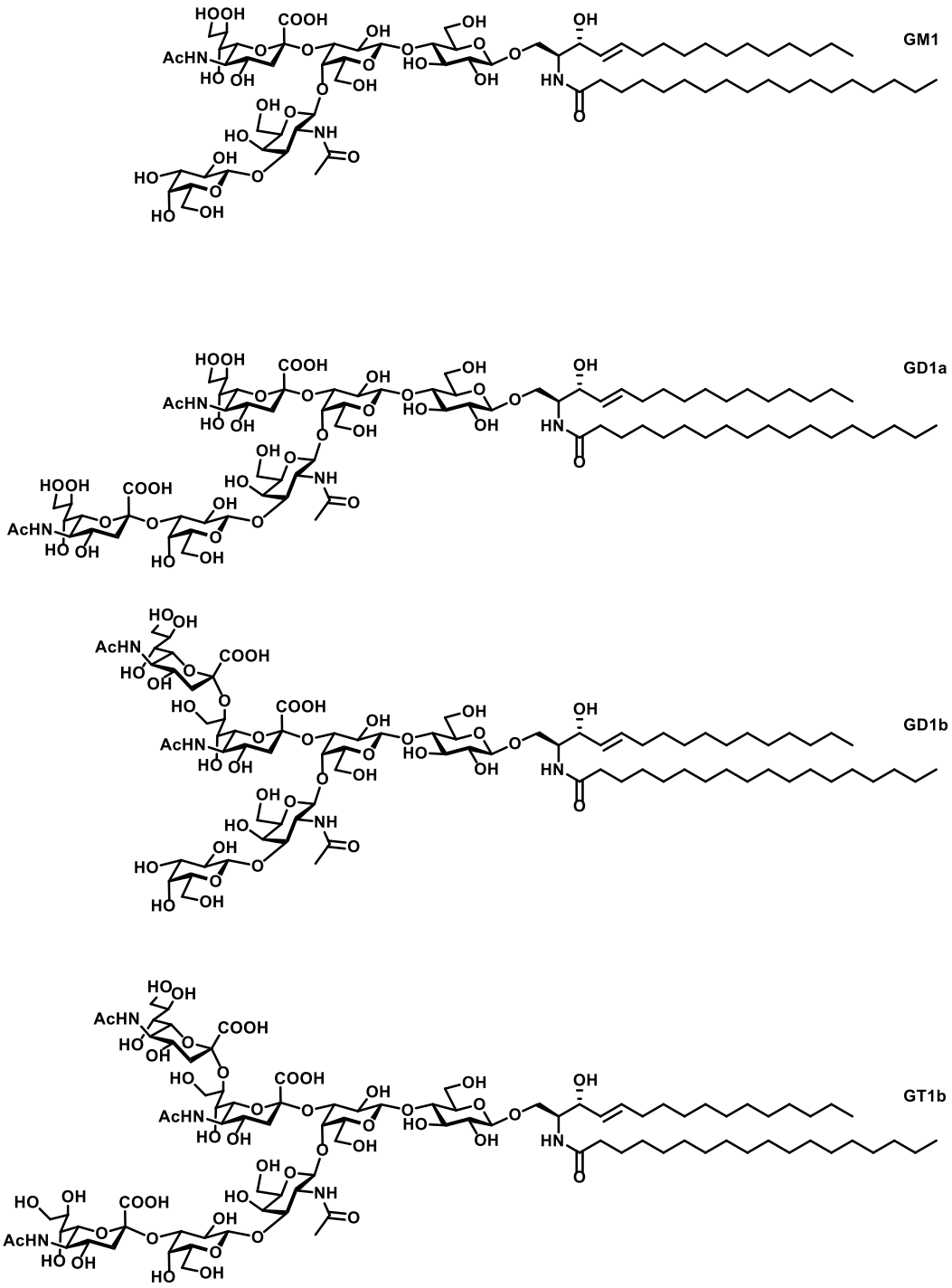


Figure 1.4. The most abundant gangliosides found in mammalian brain.[23]

1.1.4 Biological roles of gangliosides

Gangliosides are an essential component of multicellular life and participate in critical biological functions such as, but not limited to, cell signaling,[36] endocytosis,[37] and membrane receptor interactions.[38] In higher animals gangliosides are essential for maintaining a healthy nervous system.[39] Knocking out GM3 and GM2 synthases (which halts production of GM3 and GM2) resulted in severe neurodegeneration which was eventually lethal to mice.[40] The absence of GM3 and GD3 was found to be highly correlated with hearing loss in human children.[41] Gangliosides can carry blood group (ABO or ABH) antigens.[42]. GSLs carrying sialyl Lewis-X (also known as CD15s) have been proposed to be involved in leukocyte attachment to endothelial cells and modulation of immune response.[43] Gangliosides may act as receptors for the binding of microorganisms and toxins. For example, Cholera toxin binds with higher specificity to GM1,[38] and Shiga toxin binds to Gb3.[44]

GSLs are associated with genetic disorders such as Tay-Sachs disease, a lysosomal disease, due to genetic mutations in the GM2 processing enzymes resulting in the accumulation of GM2.[45,46,47] GM1 is normally degraded by β-galactosidase; however, a mutation of the enzyme causes accumulation of GM1 in cells which results in Morquio syndrome type B (mucopolysaccharidosis IVB).[48,49]

Despite extensive research into gangliosides, there are few therapies based on their biology. Pharmacological strategies targeting GSL biosynthesis have only gained prominence in the last couple of decades.[50] Several strategies to replenish the catabolic enzymes whose absence causes lysosomal storage disorders have

shown some promise, and has been used clinically with minor success.[51] Intracerebroventricular administration of HexB[52] and highly phosphomannosylated recombinant human HexA[53] have been used to reduce the amount of GM1 and GM2 gangliosides in mouse brain.

Altered expression levels of GSLs have been linked to certain human cancers. Increased expression of GM3 has been found in renal cancer,[54] leukemia,[55] and bladder cancer.[55] In contrast, GM3 has been reported to be down-regulated in colorectal,[56] leukemia,[57,58] and ovarian cancers.[59] In a renal cancer model LacCer, has been shown to be upregulated, while GM2 is upregulated in lung cancer.[60] GM1 has also be found to be upregulated in colorectal cancer.[61] Amongst the di-sialo GSLs, GD2 and GD3 have been reported to be upregulated in breast cancer,[62,63,64,65] melanoma,[66,67,68,69] and ovarian cancer[70] (GD3 only). GD1a is upregulated in prostate cancer[71] and downregulated in colorectal cancer.[61] Gangliosides have been used as cancer markers,[72] and they have been targeted in cancer therapy.[73]

These examples illustrate that GSLs have diverse levels in a range of disease, and that understanding their regulation can be challenging. Enzymes that regulate levels of GSLs, either through catabolic or anabolic mechanisms, are an underexplored avenue for drug development in any disease where GSLs may play important roles. We will highlight features of GSL biosynthesis and catabolism for the purpose of exploring this hypothesis (*vide infra*).

1.2 **Anabolic enzymes that generate GSLs.**

Biosynthesis of GSLs begins in the endoplasmic reticulum and Golgi.[74] This is a stepwise process where glycosyltransferases (GTs) use a sugar nucleotide to

glycosylate ceramide molecules. Sialyltransferases (STs) are GTs which transfer neuraminic acid (sialic acid) from CMP-sialic acid to the target glycoside.[75] There are four different sialyltransferase families reported in humans: ST3Gal, ST6Gal, ST3GalNAc, and ST8Sia.[76] Each of these enzyme classes is named for the substrate and linkage formed by the enzyme (e.g. Neu5Ac α2-3 Gal, Neu5Ac α2-6 Gal, Neu5Ac α2-3) GalNAc, Neu5Ac α2-8 Neu5Ac). Members of all four families of ST enzymes are involved in the synthesis of gangliosides. **Figure 1.5** highlights biosynthetic pathways of the major brain gangliosides and the involvement of different STs. Efforts to develop inhibitors of ST enzymes have been challenging, and only a few examples are used clinically.[77] Continued efforts in understanding the structural and substrate differences of these enzymes will likely enable the development of inhibitors with improved specificity in the future.

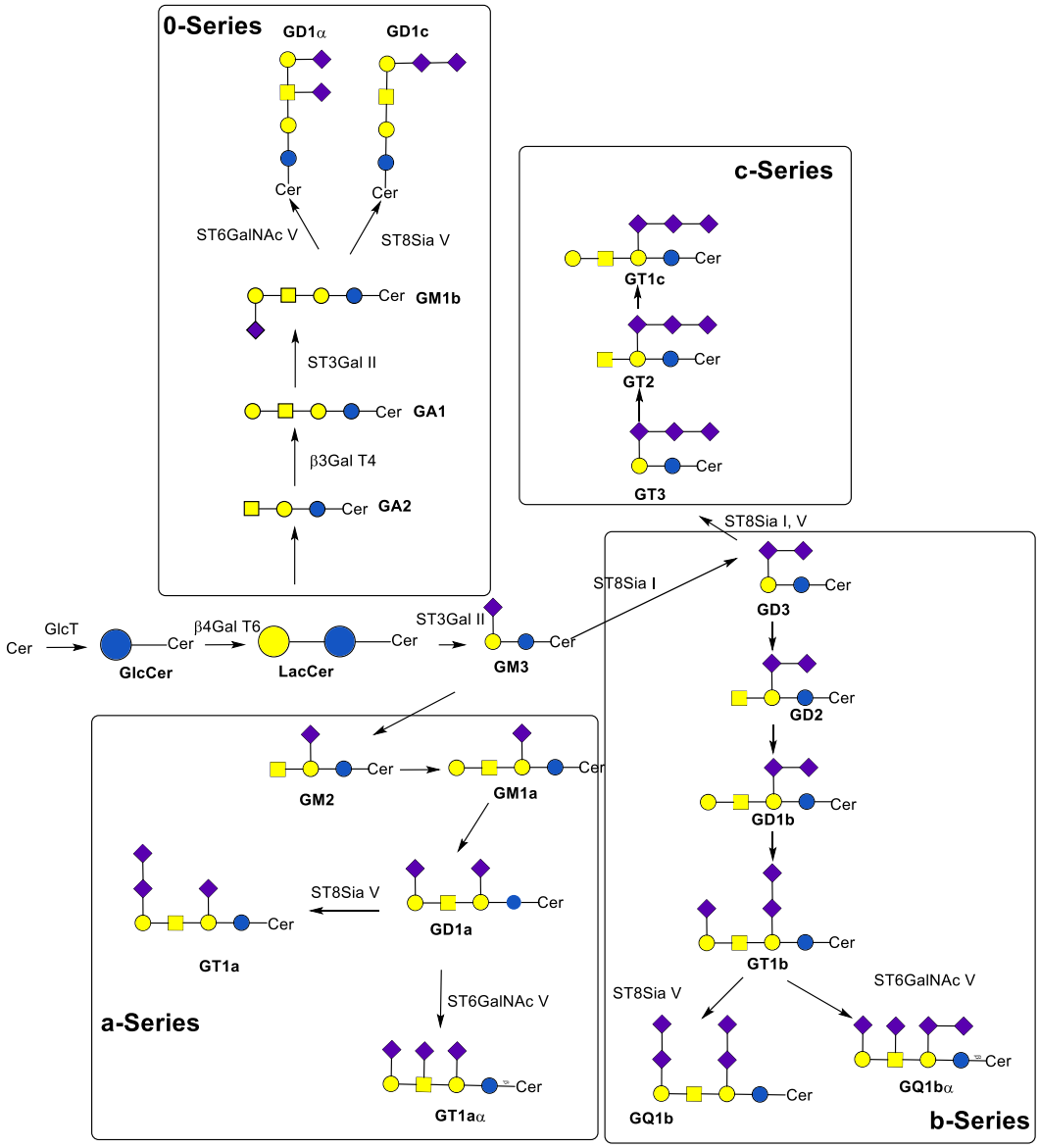


Figure 1.5. The biosynthesis of GSL by Sialyltransferases. The biosynthesis of the four major GSL classes (o, a, b, and c-series) are shown. Figure adapted from Essentials of Glycobiology.[3]

1.3 **Catabolic enzymes that modify GSLs**

GSLs are cell surface lipids that are catabolized in a stepwise fashion by at least ten different hydrolase enzymes by degradation starting at the terminal (non-reducing) end of the GSL oligosaccharide (exoglycosidases).[78] The most common enzymes involved in the catabolism are Hexosaminidase A (HexA), Galactosidase, glucocerebrosidease (GCase), and Neuraminidases (NEU). Hexosaminidase hydrolyses the glycosidic linkages of gangliosides such as GM2. Deficiency of HexA is a cause for a group of neurodegenerative disorders such as Tay-Sachs diseases.[79] Glucocerebrosidease (GCase) cleaves the glycolipid glucosylceramide (GlcCer) in lysosmal compartments and deficiencies of this enzymes cause Gaucher's diseases.[80] The β-galactosidase is an enzyme that cleaves the terminal β-linked galactose and deficiency of this enzyme causes accumulation of GM1 ganglioside,[81] a condition known as gangliosideosis.

Neuraminic acid cleaving enzymes are known as neuraminidases (NEU, or sialidases). The enzymes were first reported in 1941 by Hirst et al.[82] A purified enzyme was first reported in 1961 isolated from *Vibrio cholera*.[83] Sialidases can be classified as α-exosialidases (EC 3.2.1.18) or α-endosialidases (EC 3.2.1.129) based on whether they cleave a terminal or internal glycosidic linkage.[84] Naturally occurring enzymes are most commonly exosialidases. Endosialidase substrates must contain internal sialic acid glycosidic linkages, like those found in polysialylated compounds such as GT1b or polysialic acid polymers. All neuraminidases have a conserved sequence consisting of several Asp boxes (-Ser-X-Asp-X-Gly-X-Thr-Trp-) and the RIP (Arg-Ile-Pro) sequence.[85] The subject of

thesis will focus on the biological roles of NEU enzymes, and we will briefly summarize below the three main sources of NEU enzymes: bacterial, viral, and human.

1.3.1 Bacterial neuraminidases (bNEU)

More than 70 different microorganisms have been reported to have sialidase activity.[86] Often these microorganisms are pathogenic. Neuraminidase enzymes can function as virulence factors that may help the entry of pathogenic bacteria into host cells.[87] Many bacteria can produce more than one isoform of neuraminidase. For example, three different neuraminidase isoenzymes are produced in *Salmonella pneumonia* (NanA,[88] NanB,[89,90] and NanC[91]). Similarly, *Clostridium perfringens* expresses NanH,[92,93,94] NanI,[92,95] and NanJ.[96] The active site of the bacterial sialidase contains a glutamate residue, a pair of tyrosine residues and an aspartic residue.[90,97] The substrate specificity among different types of sialic acid linkages has been investigated for some bNEU enzymes.[98]

1.3.2 Viral neuraminidase (vNEU)

After the pathogenic nature of neuraminidase enzymes were first described in viruses, a great deal of research was performed to understand and develop drugs to target them. Influenza viral neuraminidase is ~60 kDa in molecular mass, and exists as a 240 kDa homotetramer.[99] Structurally, the enzyme resembles a mushroom-shape having a head of 80x80x40 Å and a thin structure which is 15 Å wide and anywhere from 60 to 100 Å long.[100] The influenza vNEU consists of 470 amino acids.[101] There are two major glycoproteins found in the viral particle, hemagglutinin (HA) and neuraminidase (NA).[102] Currently, 16 different types of

HA (H1 - H16) and nine different types of NA (N1 – N9) have been identified.[103] Viral neuraminidase from different host species vary in their substrate specificity. Human influenza virus shows at least three orders of magnitude difference in specificity towards α(2,3)-linked sialic acid over α(2,6)-linked sialic acid whereas, avian influenza virus shows at least one order of magnitude preference towards α-(2,6)-linked sialoglycoconjugates.[104]

Commercially available anti-influenza drugs that target the viral NA and reduce the severity of infection[105] are based on 2-deoxy-2,3-didehydro-*N*5-acetylneuraminic acid (DANA) (**1.3, Figure 1.5**). Clinically approved, anti-influenza drugs include zanamivir (**1.4**), and oseltamivir (**1.5**). Currently, the clinically available inhibitors of viral NA are inactive against the hNEU enzymes.[106]

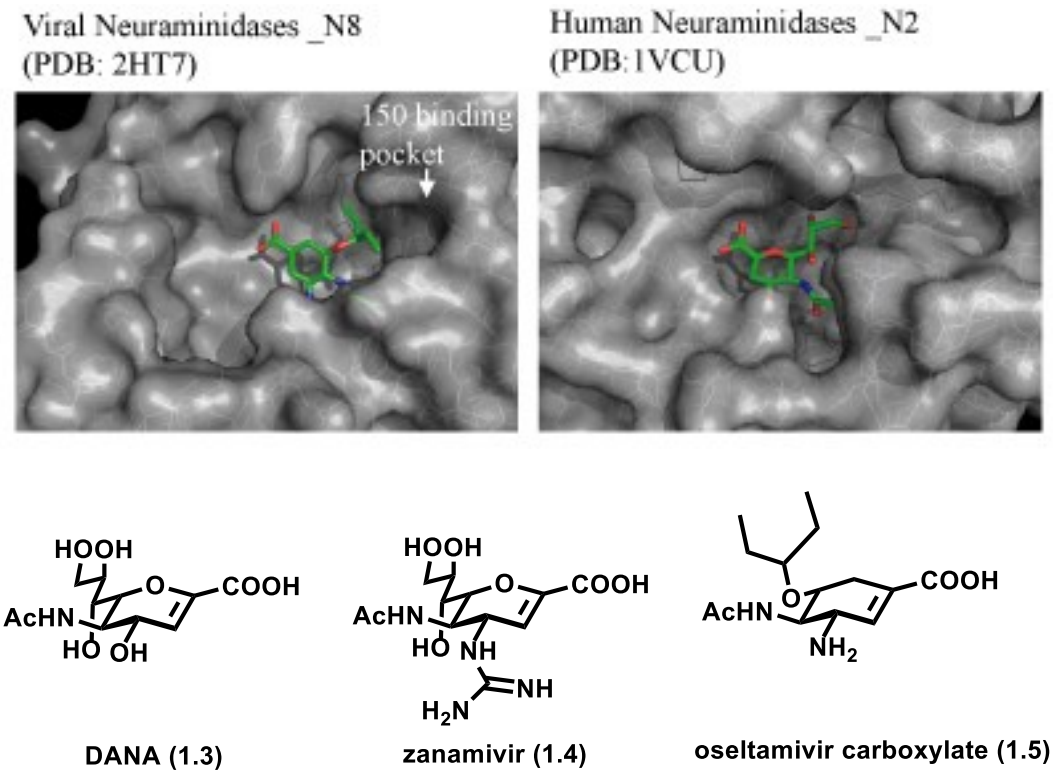


Figure 1.6. The active sites of viral and human neuraminidase enzymes. The active site of the viral and human neuraminidase shows distinct differences. (a.) The viral N8 isoform shows a distinct 150 binding pocket in its open form. The enzyme shown here is in complex with oseltamivir (PDB: 2HT7).[107] (b.) The human neuraminidase 2 (NEU2) enzyme is shown in complex with DANA (PDB: 1VCU).[108]

1.3.3 Human neuraminidase (hNEU)

Four different mammalian NEU isoenzymes have been identified, these are: NEU1, NEU2, NEU3, and NEU4.[109] **Table 1** summarizes the general properties of these four glycosyl hydrolases (GH). Neuraminidases of mammalian origin also contain the RIP (Arg-Ile-Pro-) consensus sequence and the Asp-boxes found in the viral and bacterial enzymes.[74] However, the mammalian enzymes have marked differences in terms of structure, selectivity, and specificity towards their substrates.

Table 1.1: Properties of four mammalian sialidases (adapted from Miyagi et.al.[74])

	NEU1	**NEU2**	**NEU3**	**NEU4**
Major localization	Lysosomes	Cytosol	Plasma Membrane	Lysosomes, Mitochondria, and ER Glycoproteins Gangliosides
Optimum pH	4.4-4.6	6.0-6.5	4.5-4.8 6.0-6.5	4.5-4.7
Endogenous substrates	LAMP-1 IGF-1R Integrin β4	Sialyl-Le^X GM3	GM3 GD3 GD1a	Sialyl-Le^X Polysia-NCAM GD1a
*Total amino acids**	H- 415	H- 380	H- 428	H- 496 (484)ǂ
	M- 409	M- 379	M- 418	M- 501 (478)ǂ

*, H=Human, M=Mouse; ǂ, The NEU4a and NEU4b isoforms are truncated forms.

While both viral neuraminidase and human neuraminidase cleave neuraminic acid, inhibitors of viral neuraminidase have limited activity against human neuraminidases.[110] The inhibitors of viral neuraminidase have been designed from a supposed transition-state mimic for NEU, DANA (**1.3**). Differences between enzyme isoforms, or those from different organisms, result in altered potency for inhibitors optimized against any one target (e.g. viral enzymes). Thus, it is no surprise that vNEU inhibitors have low activity against hNEU.[111] For example, vNEU inhibitors designed to take advantages of the distinct recognition pocket of viral neuraminidases (**Figure 1.6**) are not active against hNEUs. Commercially available vNEU inhibitors, such as zanamivir and oseltamivir, have weak potency against hNEU. In a recent study, zanamivir was found to have higher micromolar K_i against hNEU whereas oseltamivir activity was greater than 1 mM range.[103]

1.3.3.1 NEU1

The human lysosomal sialidase NEU1 was first reported in 1996 by Bonten et. al.[112] The NEU1 gene was initially reported in mice on chromosome 17,[113] and in humans on chromosome 6p 21.3.[74] NEU1 shares 21-28% sequence similarities between bNEU.[114] NEU1 is associated with a protective protein, Cathepsin A (CathA), and β-galactosidase (GAL), and is only active as part of this trinary complex.[115]

Sialidosis is a genetic disease that results from frameshift insertion and missense mutations of the NEU1 gene, ultimately leading to the accumulation of NEU1 substrates in the lysosomal compartment. Galactosialidosis is a

neurodegenerative disorder which occurs as a result of loss in activity for NEU1 and galactosidase due to loss of the protective function of carboxypeptidase A.[116]

The homology of NEU1 to other isoenzymes is low, with similarity of 19%-24% to hNEU isoenzymes NEU2, NEU3, and NEU4. The homology of NEU2, NEU3, and NEU4 among each other is 34%-40%.[74] NEU1 has a higher expression level than all other hNEU isoenzymes, as it is typically expressed at 10-20 fold higher levels than other isoenzymes.[117,118]

The NEU1 enzyme has been associated with a number of diverse biological functions. NEU1 has been implicated as a negative regulator of LAMP-1 mediated exocytosis.[119] NEU1 has been implicated in Lewis-X (CD15) mediated adhesion of neutrophils.[120] NEU1 may also be responsible for the production of the immune mediator, Interleukin-4 (IL-4), as T cells with low NEU1 activity failed to produce IL-4. Expression of NEU1 on the surface of T cells also increased the production of another inflammatory mediator Interferon-gamma (IFN-γ).[121] Bacterial lipopolysaccharides (LPS) failed to induce an inflammatory response in dendritic cells,[122] indicating that NEU1 is essential for defense against microorganisms. It has been suggested that NEU1 may be a target for the development of anti-asthma drugs, since NEU1-mediated desialylation of CD44 is responsible for inducing airway inflammation and hyper airway responsiveness.[123] NEU1 expression was increased 12-fold in macrophages that underwent differentiation from blood monocytes.[124] Macrophages from NEU1-deficient mice showed increased sialylation and decreased phosphorylation of FcγR and serine-tyrosine kinase.[125] These macrophages had reduced ability to engulf gram-positive and gram-negative

bacteria. A later study linked NEU1 activity to toll-like receptors (TLRs). NEU1 activity was increased when the TLRs bind with their ligands in macrophages.[126] These findings strongly suggest important functions of NEU1 in inflammation.

NEU1 has been implicated in the progression of cancer and cell migration. Miyagi *et al.* reported the metastatic potential of transformed 3Y1 rat carcinoma cells[127] and mouse colon adenocarcinoma 26 cells[128] were inversely correlated to their amount of neuraminidase activity, which was later confirmed to be NEU1. The same group showed that expression of NEU1 in B16 melanoma cells and NEU1 overexpression in HT-29 cells[129] caused the cells to become desialylated and led to reduced metastasis.[130] Overexpression of NEU1 caused decreased migration, adhesion, and invasion properties of cells; and this effect may be related to the down-regulation of matrix metalloprotease-7 or desialylation of integrin β4.[129] Inhibition of NEU1 activity resulted in increased cell proliferation of arterial smooth muscle cells, normal dermal fibroblast,[131] and insulin-treated L6 cells.[132]

Recently, NEU1 has been associated with the activation insulin receptor (IR), as NEU1-deficient mice on a high-fat diet were more likely to be obese than wild type mice.[133] Restoration of NEU1 expression was able to reverse insulin resistance in obese mice[134]. NEU1 has also been tied to the development of atherosclerosis.[135] Recently, it has been shown that ApoE KO mice with reduced activity of NEU1 reduced the serum levels of low-density lipoprotein (LDL), which significantly reduced the development of atherosclerotic plaques.[136]

1.3.3.2 NEU2 neuraminidase

The NEU2 isoenzyme was first cloned from the rat NEU2 gene, which contains two E-box pairs in the 5´-flanking region.[137] The NEU2 enzyme is found in the cytosolic compartment, and the protein is the only member of the hNEU family which is not membrane associated. As a result, NEU2 is the only mammalian neuraminidase that has reported X-ray crystallography structures. These pioneering structural studies revealed a canonical six-bladed β-propeller structure.[138] NEU2 has generally low expression levels in human cells, and may often be undetectable.[139]

NEU2 has been shown to take part in neuronal differentiation of PC12 cells[140] and myoblast differentiation in C2C12 cells.[141] These studies also indicated NEU2 involvement in activation of nerve-growth factor, which influences differentiation. NEU2 has broad activity against glycoproteins, glycolipids, and is the only neuraminidase active at neutral pH,[74] likely due to its presence in the cytoplasmic compartment.

1.3.3.3 NEU3 sialidase

Miyagi *et al.* first cloned NEU3 from bovine brain in 1999,[142] and it was subsequently cloned from a human cDNA and a genomic library.[143,144] NEU3 is present on the cell surface[142] and endosomal structures,[145] but its location may vary depending upon growth stimulus.[146] Unlike other NEU enzymes, NEU3, has two pH optimum ranges at pH 4.5-4.8, and at pH 6.0-6.5.[74] The X-ray structure of NEU3 is yet to be solved, but homology models have been proposed.[147] NEU3 prefers α(2,3), and α(2,8)-linked sialic acids and requires a hydrophobic aglycone

in the substrate for activity.[148] Phosphatidic acid has been implicated in the activation and translocation of NEU3 to the plasma membrane.[149] The Sp1/Sp3 transcription activators stimulate NEU3 expression.[150] When cultured skeletal muscle cells were subjected to hypoxic conditions, NEU3 was found to be activated, resulting in enhanced cell survival through epidermal growth factor receptor (EGFR) and the hypoxia-inducible factor1 alpha (HIP1α).[151] The environment of cancer cells is predominantly hypoxic, and these findings indicate a possible contribution of NEU3 activity in the development, spread, and prognosis of cancer.

One of the best indications of the involvement of NEU3 in cancer was published in 2015 by Miyagi and coworkers. In this study, increased serum sialidase activity was detected in prostate cancer patients, but was absent in healthy volunteers.[152] The NEU3 isoenzyme was later identified as the enzyme responsible for this increased activity. In other cancer models mRNA levels for NEU3 were found to increase in the lymph node of head and neck squamous cell carcinoma, which correlated with the progression of lymph node metastasis.[153] Relative mRNA levels of NEU3 have been found to be increased in colon cancer compared to its surrounding non-cancerous mucosa.[154] Upregulation of NEU3 has been found in prostate cancer[155] and renal cell carcinomas.[156] In renal carcinoma models, transfection of NEU3 caused the cells to become resistant to apoptosis. Silencing of NEU3 in a prostate cancer cell model resulted in decreased cell growth in culture and reduced the size of transplanted tumors in nude mice.[157] Colon cancer cells were more resistant to sodium butyrate-induced apoptosis and marked

accumulation of lactosyl ceramide (LacCer) was found after NEU3 was overexpressed.[158]

NEU3 is involved in cell invasion, motility, and metastasis. Human prostate cancer cells (PC-3) with reduced NEU3 expression showed decreased invasion and metastatic potential.[159] NEU3 was proposed to be responsible for activating matrix metalloprotease MMP-2 and MMP-9, leading to breakdown of the extracellular matrix and reduced cell migration. These findings provide a strong indication of a role for NEU3 in suppressing apoptosis and increasing metastasis of cancer cells. As a result, NEU3 may be an important new target for the development of anti-cancer drugs if compounds which can target NEU3 are identified.

1.3.3.4 NEU4 Sialidase

The NEU4 isoenzyme has been found in human[160] and mouse,[161] and was the most recently discovered isoenzyme. NEU4 is found in two forms: the long and short (truncated) form (typically referred to as NEU4a and NEU4b, respectively).[109] The long form has 12 more amino acids at its N-terminus, and this short peptide segment is believed to be a mitochondrial targeting sequence. The short form is found in the endoplasmic reticulum.[117,162]

In contrast to NEU3, mRNA levels of NEU4 were significantly reduced in colon cancers compared to surrounding non-cancerous mucosa.[154] In these same tissues, the amount of NEU1 mRNA remained almost the same. Moreover, overexpression of NEU4 in DLD-1 cells and HCT-15 cells were found to correlate with decreased cell migration, cell invasion, and increased apoptosis. Overexpression of NEU4 has been observed to suppress neurite formation, and the

level of NEU4 was decreased during retinoic acid-induced Neuro2a cell differentiation.[118]

The relation between the downregulation of NEU4 and cancer metastasis is not clear. An investigation by Tringali *et al*., found that NEU4 overexpression caused a significant increase in cell growth in neuroblastoma cells.[163] Although mRNA expression may not be an indication of actual protein expression, this group later showed that NEU4 selective inhibitors had the ability to suppress the proliferation of these cells, supporting the involvement of NEU4 in cancer progression.[164]

1.4 **Inhibitors of human neuraminidase enzymes**

Inhibitors of viral neuraminidase (vNEU) have been developed as clinical therapies against influenza. Design of viral neuraminidase inhibitors has been very successful and these compounds are widely used.[105] Zanamivir, oseltamivir, and peramivir are clinically used viral neuraminidase drugs that have excellent inhibitory activity against vNEU. However, despite high potency of these compounds for vNEU they are effectively inactive against hNEU.[110,111]

The most broadly active hNEU inhibitor is 2-deoxy-2,3-didehydro-*N*-acetyl-neuraminic acid (DANA, **1.3**). The compound is a dehydration product of Neu5Ac,[165] and is even produced by NEU enzymes from Neu5Ac. Most rationally-designed hNEU inhibitors are based on the DANA scaffold. The first selective hNEU inhibitor reported was the C9-modified *n*-pentyl amide with activity against NEU1,[166] and an IC_{50} value of 10 μM.

Our group has been working to develop selective inhibitors of the hNEU isoenzymes using rational drug design strategies.[167] Currently, nanomolar potency inhibitors for NEU1, NEU3, and NEU4 have been discovered. The most potent and nanomolar-selective NEU4 inhibitor was reported with a K_i = 30 ± 19 nM. This compound was produced with a triazole modification of the C9 position of DANA.[168] Our group also identified a 12-fold selective inhibitor for NEU2 (IC_{50} 86 ± 17 μM) and 40-fold selective compound for NEU4 (IC_{50} 24 ± 2 μM) by screening a library of C4 and C7 modified DANA derivatives.[169] More recently, through modification of C4 and C9 positions of DANA, the first nanomolar-selective NEU3 inhibitor with a K_i = 320 ± 40 nM was identified.[170] Most recently, the first nanomolar-selective NEU1 inhibitor was synthesized as C5-hexanamido-C9-acetamido-DANA with a K_i = 53 ± 5 nM.[171]

1.5 **Modulation of cell adhesion receptors**

Integrins are heterodimeric glycoproteins found on the plasma membrane that play a major role in cell adhesion.[172, 173] Each heterodimer consists of two glycoprotein subunits known as the α and β subunits. There are 18 different α units and 8 different β units discovered to-date in humans.[174] Specific heterodimers may be referred to by the name of the individual subunits, or their corresponding CD antigen names. For example, a lymphocyte integrin originally identified as critical to lymphocyte function was first named as the "lymphocyte functional antigen-1" (LFA-1); but is now also referred to as the $\alpha_L\beta_2$ integrin, or CD11a/CD18 (where the CD antigen refers to each integrin subunit). Crystal structures of the $\alpha_v\beta_3$ integrin revealed that integrin functionality is conformation-dependent, and these

heterodimers can adopt different conformations to carry out their functions.[175] Integrins regulate adhesion by binding with components of the extracellular matrix (ECM) such as laminin, fibronectin, and collagen.[176] When they bind with ECM, integrins can switch conformations from low-affinity (inactive state) to high-affinity (active state), or vice-versa.[177,178] Most integrins are tethered directly or indirectly to cytoskeletal components and can regulate cytoskeletal remodeling through actin polymerization.[176,179]

Like most membrane proteins, integrins are not fixed in place - but rather diffuse through the plasma membrane.[180] When integrins bind with ECM, the resulting complex may contain upwards of 80 different kinds of proteins and subsequent clustering of the integrin complex results in formation of a *focal adhesion complex* ranging in size from 250 nm to >5 μm.[181] Integrins are essential for life, and deletion or mutation of integrin genes often results in fatal consequences.[174] The β2-integrin, LFA-1, is only found in leukocytes. When activated through cytokine-induced mechanisms, LFA-1 becomes activated and enables circulating leukocytes to bind to endothelial cells and migrate to the site of inflammation. LFA-1 binds hight affinity with ICAM-1 and ICAM-3. LFA-1 KO mice lacking the β2-integrin developed spontaneous ulceration and had defective T cell function.[182] Patients suffering from leukocyte adhesion deficiency type I (LAD I) due to mutation of the β2 gene suffer from leukocytosis.[183] The structure, function, and mechanism of individual integrins are complex, and a detailed discussion is beyond the scope of this thesis.[176,184]

The hNEU enzymes have previously been implicated in the regulation of cell adhesion. However, specific regulation of receptors by hNEU isoforms has not been clearly established. Integrins are remarkably sensitive to conformational change, are often found in membrane microdomains, and are heavily glycosylated. Each of these factors have the potential to be influenced by NEU activity. One objective of this thesis was to investigate biochemical links between integrins and hNEU.

1.6 **Hypothesis and objectives**

Based on our review of the literature and previous work from our group, we hypothesized that neuraminidase enzmyes could act as biochemical regulators of sialic-acid dependent processes. Processes regulated by NEU could include glycoprotein conformational changes, altered binding affinities, or changes to glycolipid composition within the membrane. In our initial studies of hNEU regulation of integrins, we observed that β1 integrins could be blocked by hNEU inhibitors.[185] In this thesis, we sought to expand on these findings to investigate the role of specific hNEU isoenzymes, and also to examine different integrin receptors that could be important for health and disease.

The central hypothesis of this thesis is that hNEU enzyme activity modulates the activity of integrin receptors and influences integrin-dependent processes. Furthermore, we propose that individual hNEU isoenzymes may play different, or even opposing, roles in integrin regulation. Finally, we hypothesize that if specific hNEU regulation of an integrin can be identified, selective inhibitors of isoenzymes could be used as therapeutic tools. The specific objectives of each chapter in this thesis are summarized below.

In Chapter 2, we investigate the influence of hNEU on the adhesion of T cells using the β2 integrin, LFA-1. Integrin-mediated adhesion of leukocytes to the vascular wall is already known to be dependent upon sialylated antigens, such as sialyl-Lewis X (CD15s). However, we wanted to investigate the role of hNEU isoenzymes, such as NEU3, in altering the activity of LFA-1 and its ability to bind its ligand, ICAM-1.

In Chapter 3, we investigated the role of hNEU in regulating the migration of cancer cells *in vitro*. We specifically investigated the migration of cells on FN-coated surfaces, which involves the α4β1 and α5β1 integrins. We used a cell migration assay to confirm that specific hNEU isoenzymes regulate integrin-mediated cell migration on FN. Importantly, we tested the utility of isoenzyme-selective inhibitors in this system and observed that hNEU isoenzymes can play distinct and opposing roles in this system. These results have important implications for the future development of anti-metastatic and wound-healing therapeutics.

In Chapter 4, we investigated the role of hNEU isoenzyme regulation of inflammation *in vivo*. Using a murine model of inflammation, we observed changes in leukocyte recruitment to a site of inflammation after disruption of hNEU expression. Animals with reduced or absent expression of NEU1, NEU3, and NEU4 showed distinct changes in leukocyte recruitment to the site of inflammation. These are the first robust studies of hNEU influence upon leukocyte recruitment and could form the basis of future anti-inflammatory therapeutics.

Finally, in Chapter 5 we summarize the major conclusions of this work and prospects for future investigations of hNEU biochemistry.

1.7 **References**

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2. Neuraminidase-3 can act as a negative regulator of LFA-1 adhesion[1]

[1] Compounds used in these studies were synthesized and characterized by Yi Zhang, and Tianlin Guo; Caishun Li performed SPT, imaging, homotypic aggregation, and HPTLC experiments; Radhika Chakraberty performed LC-MS analysis of glycolipids; Chunxia Zou performed in vitro adhesion assays. Md. Amran Howlader conducted experiments, analyzed data, wrote manuscripts. A version of this chapter has been published in Frontiers in Chemistry, 22 November 2019. https://doi.org/10.3389/fchem.2019.00791.

2.1 **Introduction**

The process of leukocyte rolling, extravasation, and homing to sites of inflammation is critical to cellular immunity, and is known as the leukocyte adhesion cascade.[1] Along each step of this process different cell adhesion molecules and their ligands mediate recognition between leukocytes and endothelial cells. The initial attachment of the leukocyte to the endothelial wall, usually referred to as rolling, is mediated by selectins and their carbohydrate ligands (e.g. sialyl Lewis-X; CD15s).[2] Later steps of the process must arrest the cell (firm adhesion) to allow for transmigration. These later steps of the process are largely mediated by integrin receptors and their ligands. Integrins are a major class of adhesion receptors and an important therapeutic target.[3,4,5] The first integrin in the inflammatory cascade is LFA-1 (known as the αLβ2 integrin; or CD11a, CD18), a transmembrane receptor which binds to ICAM-1 (inter-cellular adhesion molecule-1; CD54) and conveys an outside-in intracellular signal to the leukocyte.[6] These and subsequent integrin-mediated processes, including interactions of the very-late antigens (VLA-4, the α4β1 integrin or VLA-5, the α5β1 integrin), allow cells to migrate to the site of inflammation.[3,7,6] Thus, processes which modulate leukocyte integrin function are of potential interest for the development of anti-adhesive and anti-inflammatory therapeutics.[3,7,6]

Cell surface glycoconjugates are critical components of the plasma membrane. Amongst them are sialic acid-containing glycolipids, known as gangliosides, which also has important structural and functional roles. Sialic acid (also known as neuraminic acid, or Neu5Ac) has long been recognized to participate in the

regulation of immune cell function. The sialic acid content of lymphocyte receptors is known to be altered as part of cell development,[8] infection,[9] and activation.[10] The enzymes that remove sialic acid, known as neuraminidases (NEU; also called sialidases), increase trans-endothelial migration,[11] reduce expression of CD15s,[12] and expose integrin activation epitopes.[13] Early reports dubbed increases in B cell antigen sensitivity a "neuraminidase effect,"[14] and recent evidence has ascribed this phenomenon to sialic acid acting as a negative regulator of immune cell interactions.[15] The prominent role of sialic acid in adhesion suggests that changes which affect sialoglycoconjugates (SGC) may be critical to regulation of cell-cell interactions.

Catabolic remodeling of glycoconjugates is likely to be more rapid than biosynthetic processes.[16] Membrane-associated glycosyl hydrolase (GH) enzymes could play a role in signaling pathways through processing of glycolipids or glycoproteins. This hypothesis is consistent with the increased turnover of terminal glycan residues (e.g. sialic acid and fucose) relative to core glycan residues,[17] and the rapid loss of sialylated antigens on neutrophils.[12] The family of human neuraminidases (hNEU) have been shown to participate in a variety of signaling pathways and pathologies including inflammation, adhesion, tumorigenesis, and cancer metastasis.[18, 19] However, the role of specific hNEU isoenzymes has not been well defined within inflammation.

The NEU3 isoenzyme is known as a plasma-membrane-associated GH which has a strong preference for glycolipid targets.[20-23] Additionally, the enzymatic activity of NEU3 is modulated by signaling events such as protein kinase C

stimulation in immune cells.[24] The specificity of NEU3 for glycolipids, and its localization to membrane microdomains,[25] suggests a central role for the enzyme in cellular signaling.[22] The glycolipid GM3 is a key component of lipid rafts, as well as a substrate for NEU3.[26] Our group has been interested in the function of NEU3 in regulating membrane organization. We wanted to investigate the effects of NEU3 on integrin-mediated leukocyte adhesion through its regulation of SGC. Glycolipid interactions with integrins have been examined by a number of groups.[27] Lactosyl ceramide (LacCer) has been shown to activate β1 integrins.[28, 29] The activation of LFA-1 in neutrophils has been found to require LacCer enriched domains.[29] Near-field imaging studies have found that LFA-1 on resting cells is not associated with glycolipid-enriched domains; however, activation of cells allows LFA-1 nanodomains to assemble into larger clusters with glycolipid-associated proteins.[30] Taken together, these reports suggest an important role for glycolipids in the regulation of integrin organization and function on lymphocytes.

In this study, we investigated the role of the human NEU3 isoenzyme in regulating LFA-1 adhesion in a T cell model (Jurkat) and peripheral blood mononuclear cells (PBMC). We found that the enzyme altered the glycolipid composition of cells, as well as the organization of LFA-1 in the membrane. By measuring the lateral mobility of LFA-1, we provide mechanistic insight into the altered distribution of LFA-1. We observed that NEU3 activity significantly increased LFA-1 lateral mobility and endocytosis, and blocked LFA-1–ICAM-1 adhesion. We also found that NEU3 treatment did not block all adhesion pathways, as homotypic aggregation of cells was increased. Together, our results support a

role for NEU3 in the regulation of lymphocyte integrins and may implicate the enzyme in the inflammatory cascade.

2.2 Results

2.2.1 NEU3 treatment reduced sialylated-glycolipids in cells

To gain insight into gross changes in the composition of membrane glycosphinolipids (GSL), we first employed high-performance thin layer chromatography (HPTLC). Jurkat T cells were treated with conditions expected to alter integrin function, and GSL were extracted and analyzed by HPTLC.[31] We observed only minor variations in sialo- and asialo-forms of gangliosides which were difficult to quantitate (data not shown). To provide more quantitative insights we implemented an LC-MS-FLD analysis of gangliosides based on previous reports, and focused on changes to the ratio of LacCer to GM3 (**Figure 2.1a**).[32, 33] This analysis showed no significant changes on treatment with phorbol 12-myristate 13-acetate (PMA; a protein kinase C activator) which was used as positive control, and minor, but not significant, changes with cytochalasin D (cytoD; a cytoskeletal disruptor).[34] CytoD was used to probe for its influence in glycolipid modulation as this compound was used as a negative control in migration studies described later. Human cell types typically express multiple isoforms of NEU.[19] In order to probe the role of a single NEU isoenzyme in cells, we treated cells with recombinant NEU3 enzyme and a bacterial NEU from *Clostridium perfringens* (NanI).[35, 36] We found that treatment with NanI had no detectable effect on glycolipid composition; however, NEU3 showed a significant increase in asialo

forms of GM3 (**Figure 2.1b**). This result suggested that NanI did not substantially alter ganglioside composition, while NEU3 showed more specific activity for glycolipid substrates.[26, 37] We concluded that treatment of cells with NEU3 resulted in an altered composition of membrane glycolipids, which included reduction in GM3 and an increase in LacCer.

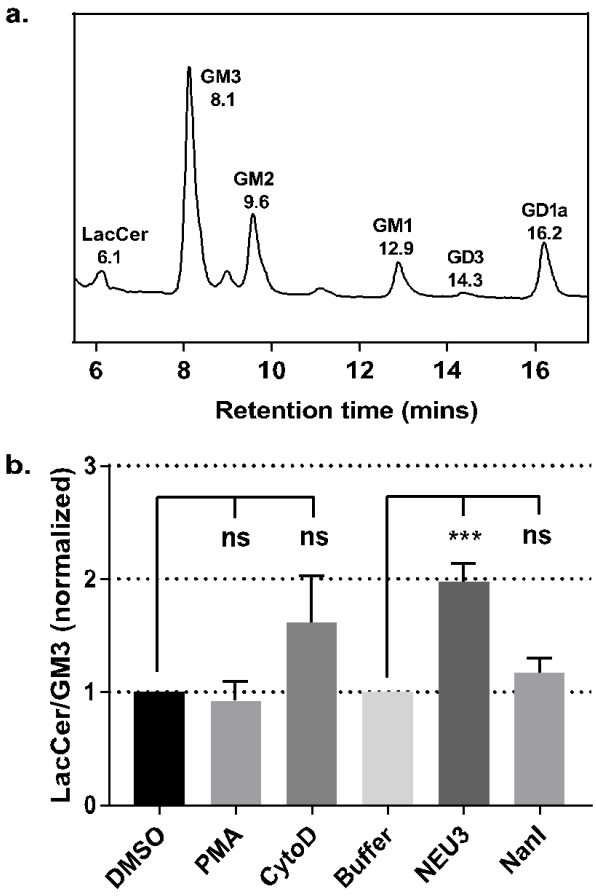

Figure 2.1. Analysis of the change of cell membrane GSLs. GSLs were extracted from treated or control cells and analyzed by LC-MS. (a.) Glycolipids extracted from Jurkat cells were digested with endoglycoceramidease, labelled and resolved by LC-MS-FLD. The major glycolipids observed were LacCer, GM3, GM2, GM1, and GD1a. (b.) LC-MS-FLD analysis was performed on four replicate samples (N = 4) for Jurkat cells treated as indicated. The ratio of LacCer to GM3 was calculated using the peak areas for each condition and normalized to the respective control. Data were compared to the indicated control using a student t-test to determine p values; ***, $p \leq 0.005$; ns, not significant.

2.2.2 NEU3 treatment altered the glycosylation of LFA-1

We used lectin blotting to detect changes in the glycosylation state of LFA-1 after NEU treatment (**Figure 2.2**). We selected the *Sambucus nigra* agglutinin (SNA), peanut agglutinin (PNA), and *Maackia amurensis* agglutinin (MAA) for this analysis. The PNA lectin binds terminal galactose residues, while SNA and MAA bind to terminal sialic acid residues.[38] We observed that treatment of purified LFA-1 with NEU3 and NanI resulted in a significant decrease in SNA and MAA staining for LFA-1, consistent with loss of sialic acid. Treatment with either NEU enzyme gave a corresponding increase in PNA staining, suggesting a corresponding increase in terminal galactose residues after loss of sialic acid. These results were consistent for both the α- and β-chains of LFA-1. Together, these data are consistent with desialylation of the LFA-1 complex, leading to an increased amount of exposed galactose sites in the presence of NEU3 or NanI activity.

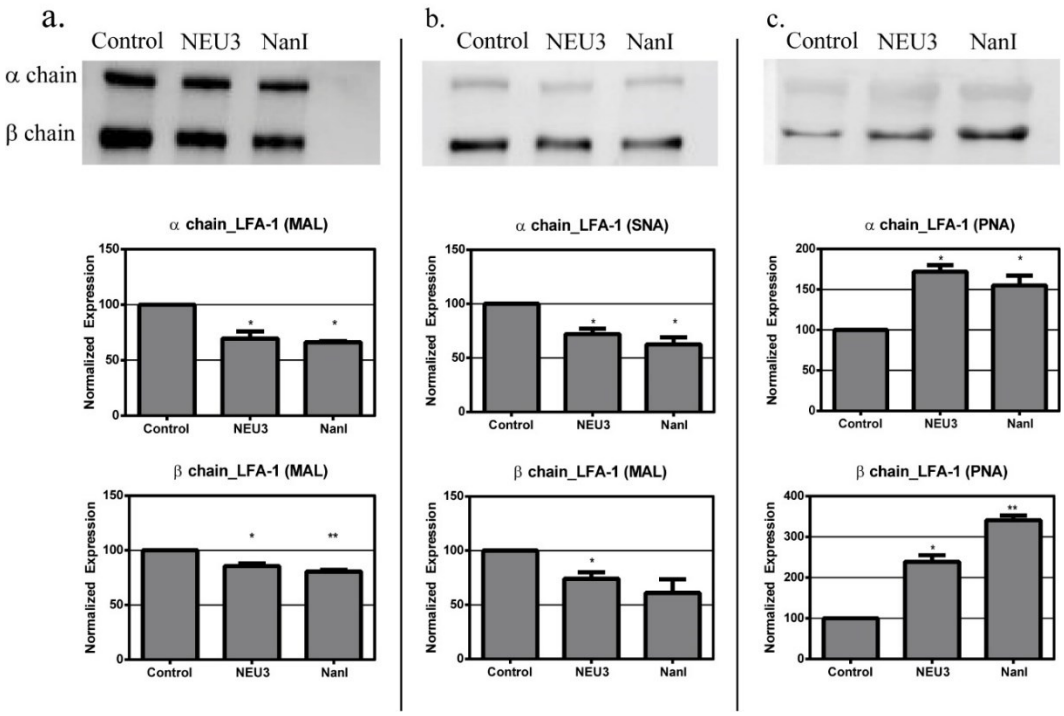

Figure 2.2: Lectin blotting of LFA-1 shows sensitivity of LFA-1 to NEU treatment. Purified LFA-1 was treated with NEU3 and NanI for 3 h at 37 °C. The protein was then used for a western blot with biotinylated lectins. Lectins (a.) MAA, (b.) SNA, and (c.) PNA were used. MAA and SNA recognize terminal sialic acid residues, while PNA recognizes terminal galactose residues. Chemiluminescent blots were developed and analyzed for changes in band intensities, and a representative image from two experiments are shown at the top of each panel (see supplementary information). Data in the plots are shown as mean ± SEM and were compared to the appropriate control using t-test to determine p values; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.005$.

2.2.3 Fluorescence imaging of LFA-1

We next sought to determine if NEU3 treatment of cells would result in changes to the localization of LFA-1. Cells were imaged by total internal reflection fluorescence (TIRF) microscopy, limiting visualization to portions of the cell in close apposition to the glass surface. Cells were stained with a Cy5-conjugated anti-LFA-1 antibody (clone TS2/4) and a FITC-conjugated Cholera Toxin subunit B (CTB-FITC) to visualize gangliosides.[39] Untreated cells showed relatively diffuse LFA-1 microclusters, while CTB gave diffuse staining and large patches with partial LFA-1–CTB colocalization (**Figure 2.3a**). Treatment of cells with NEU3 resulted in more punctate CTB staining and more diffuse LFA-1 microclusters. In contrast, NanI treatment resulted in larger co-localized regions of LFA-1 and CTB staining. A portion of the localized aggregates appeared at cell-cell contacts. Treatment of cells with PMA resulted in larger and more distinct microclusters of LFA-1 and minimal CTB colocalization (**Figure 2.3b**). Treatment of cells with cytoD disrupted CTB-positive aggregates and reduced co-localization with LFA-1 microclusters. LFA-1 is known to form nanoclusters on resting and activated cells, and the membrane domains in which LFA-1 is found tend to be heterogeneous.[40, 41] We also note that CTB staining may include reactivity to glycoprotein antigens, and therefore imaging results with this stain should be interpreted with caution. However, previous reports have suggested that GM1 is the major CTB reactive glycoconjugate in Jurkat cells.[42]

To quantitate changes in LFA-1 cluster size, we analyzed TIRF images of individual cells (N = 15) from each condition by determining the amount of LFA-1 found in clusters. Images were processed in ImageJ to identify clusters and to

determine the total area per cell found within them (**Figure 2.4, Table 2.1**).[43] The distribution of total cluster area per cell is shown in **Figure 2.4a**. The area of the cluster size are listed in Table **2.1**.

Clear increases in cluster size were observed for cytoD, NEU3, and NanI treatments. Treatment with NanI showed the largest increase in cluster area (consistent with **Figure 2.3**). Our observation that NEU3 has similar effects to cytoD in both lateral mobility and clustering indicated that enzyme activity influenced cytoskeletal regulation of the receptor.[44, 45]

Table 2.1: LFA-1 Cluster Size by TIRF

Condition	n	Cluster size [μm^2 $cell^{-1}$]	p	n	Cluster size† [μm^2 $cluster^{-1}$]	p
DMSO	15	21.2 ± 1.2	-	126	2.0 ± 0.2	-
PMA	15	22.7 ± 1.2	0.4	122	2.4 ± 0.2	0.08
cytoD	15	25.6 ± 1.4	0.025	121	2.7 ± 0.3	0.015
buffer	15	16.7 ± 1.2	-	104	1.9 ± 0.2	-
Neu3	15	22.4 ± 1.6	0.008	110	2.6 ± 0.3	0.08
NanI	15	35.7 ± 2.6	< 0.0001	137	3.4 ± 0.4	0.004

*, p values were calculated using a t-test to the appropriate control (DMSO or buffer). †, Size of individual clusters was calculated after filtering out clusters smaller than 4 $pixels^2$ (0.07 μm^2). Error is given as the standard error of the mean.

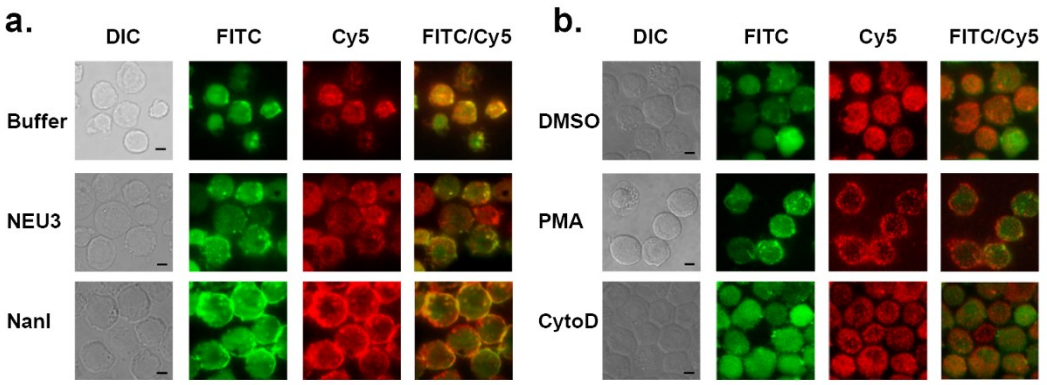

Figure 2.3. TIRF imaging of LFA-1 on treated cells. Jurkat cells were fixed after treatment with (**a.**) buffer (control), NEU3, or *NanI*; and (**b.**) DMSO, PMA, or cytoD. The fixed cells were then labelled with Cholera toxin subunit B (CTB-FITC) to label gangliosides and a TS2/4-Cy5 conjugate to label LFA-1. Cells were imaged by DIC and TIRF. Merged FITC and Cy5 images are shown in the last column with yellow indicating co-localization. Scale bar = 5 μm.

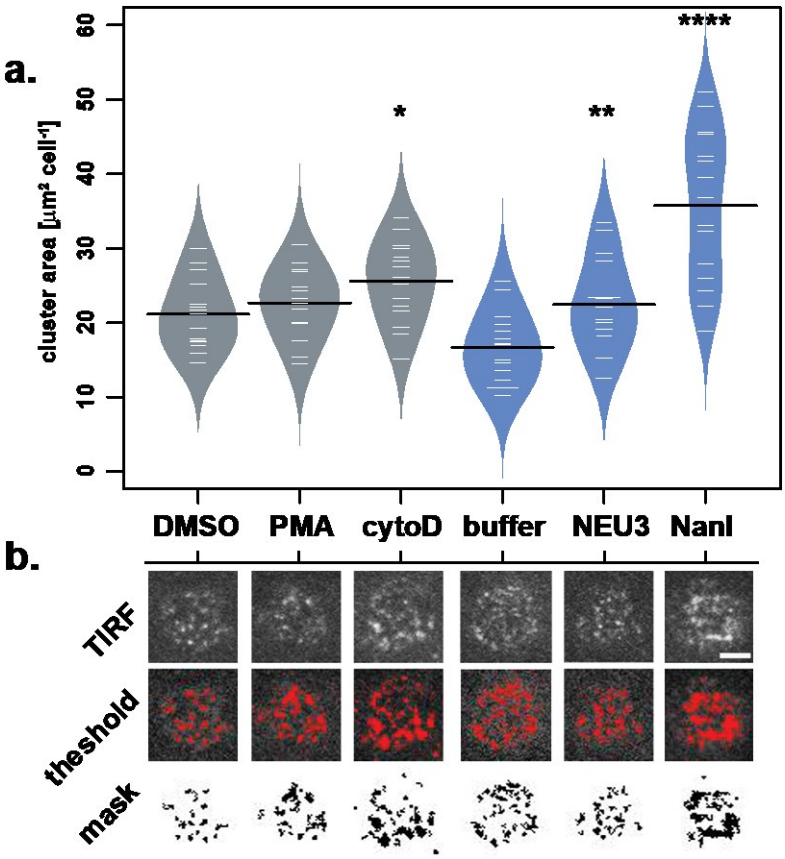

Figure 2.4. Changes in LFA-1 clustering after neuraminidase treatment. The amount of clustered LFA-1 on individual cells was determined using TIRF imaging. Cells were labeled with a TS2/4-Cy5 antibody conjugate, and 15 individual cells were compared for each condition. (**a.**) Clusters that were larger than 4 pixel^2 (0.7 mm^2) were measured using ImageJ, and the average area was tabulated for each cell. Data are plotted using a beanplot, with solid lines representing the mean of each population.[46] (**b.**) Representative TIRF images for individual cells are shown from each condition. A mask was generated from the TIRF image using a threshold for quantitation. Data were compared to the appropriate control using a t-test to determine p values; *, $p \leq 0.05$; **, $p \leq 0.01$; ****, $p \leq 0.0001$. Scale bar = 5 µm.

2.2.4 Lateral mobility of LFA-1 was altered by NEU3 treatment

We next examined the lateral mobility of LFA-1 on Jurkat T cells using single-particle tracking (SPT) methods.[47-49] Cells were labeled with Cy5-conjugated anti-LFA-1 (clone TS2/4) at low enough concentrations to achieve sparse labeling of the receptors as observed by TIRF. Videos were recorded and analyzed to determine trajectories of LFA-1 on live cells (10 sec, 10 FPS). This strategy allowed us to obtain many trajectories rapidly; however, due to photobleaching, trajectories recorded in this experiment tend to be shorter than those obtained from tracking of polystyrene beads or quantum dots. Trajectories were analyzed with u-Track and processed with custom scripts in MATlab.[45, 48] Data were pooled from multiple cells for each condition and are summarized in Table **2.2**.

Table 2.2. Diffusion of LFA-1 determined using SPT

		D_{micro}†			
Condition	N	mean (linear)	median (linear)	mean (log transformed)	median‡ (log normal)
DMSO (control)	321	5.2 ± 0.3	3.28	2 ± 1	2.2 ± 0.4
PMA	334	5.9 ± 0.4	4.31	2 ± 1	2.4 ± 0.5
cytoD	422	7.7 ± 0.7**	4.32	3 ± 1**	3.0 ± 0.5
buffer (control)	294	6.1 ± 0.6	3.28	2 ± 1	1.8 ± 0.4
NEU3	210	11 ± 1****	6.11	4 ± 1****	4.3 ± 0.9
NanI	216	5.5 ± 0.4	3.32	2 ± 1	2.3 ± 0.5

Data was analyzed using u-Track [48] and custom scripts implemented in Matlab.[45] Values listed are either the arithmetic mean, arithmetic median, or the median determined for a log normal distribution (see Supporting information.) Error is given as the standard error of the mean. †, Diffusion coefficients are in units of x 10^{-10} [cm^2s^{-1}] or x 10^{-2} [μm^2s^{-1}]; Data were compared to the appropriate control using a t-test to determine p values; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.005$; ****, $p \leq 0.0001$. ‡ Median calculated based on a lognormal fit as described in supporting information.

All diffusion measurements were calculated as micro-diffusion coefficients (D_{micro}) due to the short duration of the trajectories.[50] Our observations were in general agreement with SPT studies of fusion-protein labelled LFA-1.[51] In previous SPT observations of LFA-1 at high time resolution the diffusion coefficients were found to have a non-normal distribution.[45] We found this to also be the case in our SPT data set, as the measured diffusion coefficients spanned up to four decades. Therefore, we proceeded to analyze these data as normal and lognormal

distributions. Comparisons of the linear and logarithmic means found that LFA-1 on cytoD- and NEU3-treated cells exhibited significantly increased diffusion. Beanplots showing the distribution of diffusion coefficients are shown in **Figure 2.5**, and illustrate the shifts in LFA-1 diffusion in logarithmic scale.[46] Further analysis of these data as cumulative distribution functions (CDF) illustrate the clear increase in LFA-1 diffusion upon NEU3 treatment. Our measurements were in general agreement with previous studies of LFA-1 lateral mobility using other methods.[52] These data allowed us to conclude that NEU3 had a significant positive effect on the lateral mobility of LFA-1. Interestingly, the bacterial neuraminidase, NanI, had no significant effect on LFA-1 mobility in this experiment. These data support a specific role for NEU3 enzyme activity in the regulation of integrin mobility.

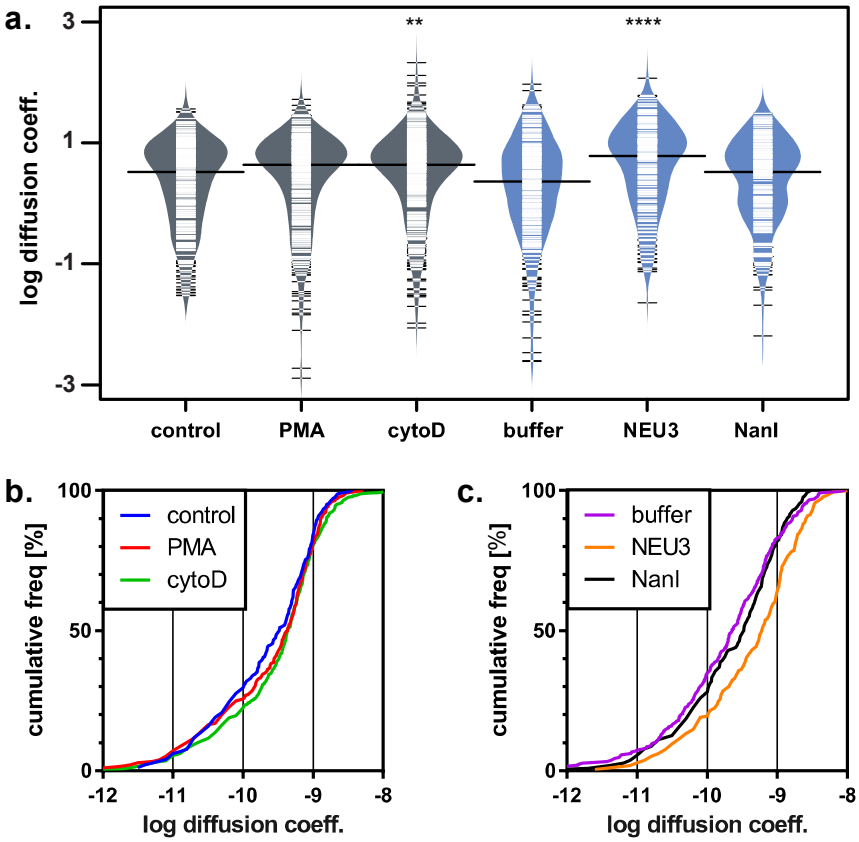

Figure 2.5: LFA-1 diffusion is altered by NEU treatment. The lateral mobility of LFA-1 on Jurkat was determined using SPT. (**a.**) A beanplot of each population is shown with the logarithmic median of diffusion coefficients indicated by a solid line for each population (same data as in Table 1.)[46] Each population is shown with a density estimate and horizontal lines indicate individual diffusion coefficient measurements. (**b.**) A cumulative frequency distribution is shown for the control, PMA, and cytoD conditions. (**c.**) A cumulative frequency distribution is shown for the buffer, NEU3, and NanI conditions. Data in c. & d. were also fit to a lognormal Gaussian population distribution (dotted lines). Diffusion coefficients are given as log(D), where D is in units of x 10^{-10} [cm^2s^{-1}] or x 10^{-2} [μm^2s^{-1}]. Data were compared to the appropriate control using a t-test to determine p values; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.005$; ****, $p \leq 0.0001$.

2.2.5 LFA-1–ICAM-1 adhesion was blocked by NEU3

To determine if NEU3 had a functional effect on LFA-1–mediated adhesion we employed a flow cytometry-based assay, similar to previous reports (see Experimental Methods).[53] Fluorescent polystyrene beads were coated with recombinant ICAM-1, and cell–bead conjugates were detected by flow cytometry. LFA-1 binding was normalized bovine serum albumin (BSA) coated beads. ICAM-1 coated beads as a positive control and bovine serum albumin (BSA) coated beads as a negative control.

We found that PMA treatment of Jurkat cells increased adhesion as expected (**Figure 2.6a**). To test the role of native neuraminidase enzymes, we treated the cells with a general neuraminidase inhibitor, 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid (DANA). DANA is known to inhibit multiple human NEU isoenzymes,[54] and we observed a significant increase in LFA-1 adhesion after DANA treatment in the absence and presence of PMA. This result was consistent with a role for native NEU activity that negatively regulates LFA-1–ICAM-1 adhesion. Treatment of cells with purified NEU3 or NanI resulted in a dramatic block of LFA-1–ICAM-1 adhesion (**Figure 2.6b**). Experiments with a NEU3(Y370F) mutant confirmed that the effect of NEU3 was due to its enzymatic activity (**Figure 2.7**).[35] Furthermore, to resolve the likely substrate of each enzyme we performed additional controls. Control experiments with ICAM-1 beads pre-treated with either NEU3 or NanI found that NEU3 treatment had no effect on ICAM-1–LFA-1 adhesion; while treatment of the same beads with NanI resulted in a large decrease in adhesion. Thus, we concluded that the two enzymes inhibited adhesion through modification of different substrates: Treatment with NanI

blocked adhesion as a result of desialylation of the ICAM-1 ligand, while NEU3 blocked adhesion through enzymatic modification of a cell surface target.

We further confirmed our observations beyond model cells by testing the effect NEU3 on ICAM-1 adhesion in PBMC (**Figure 2.6c**). Treatment of peripheral blood mononuclear cells (PBMC) isolated from healthy human donors with NEU3 or NanI enzymes resulted in a significant decrease in ICAM-1 adhesion. Notably, NEU3 treatment of Jurkat and PBMC cells partially suppressed PMA-activated adhesion suggesting a regulatory role for the enzyme late in the activation pathway.

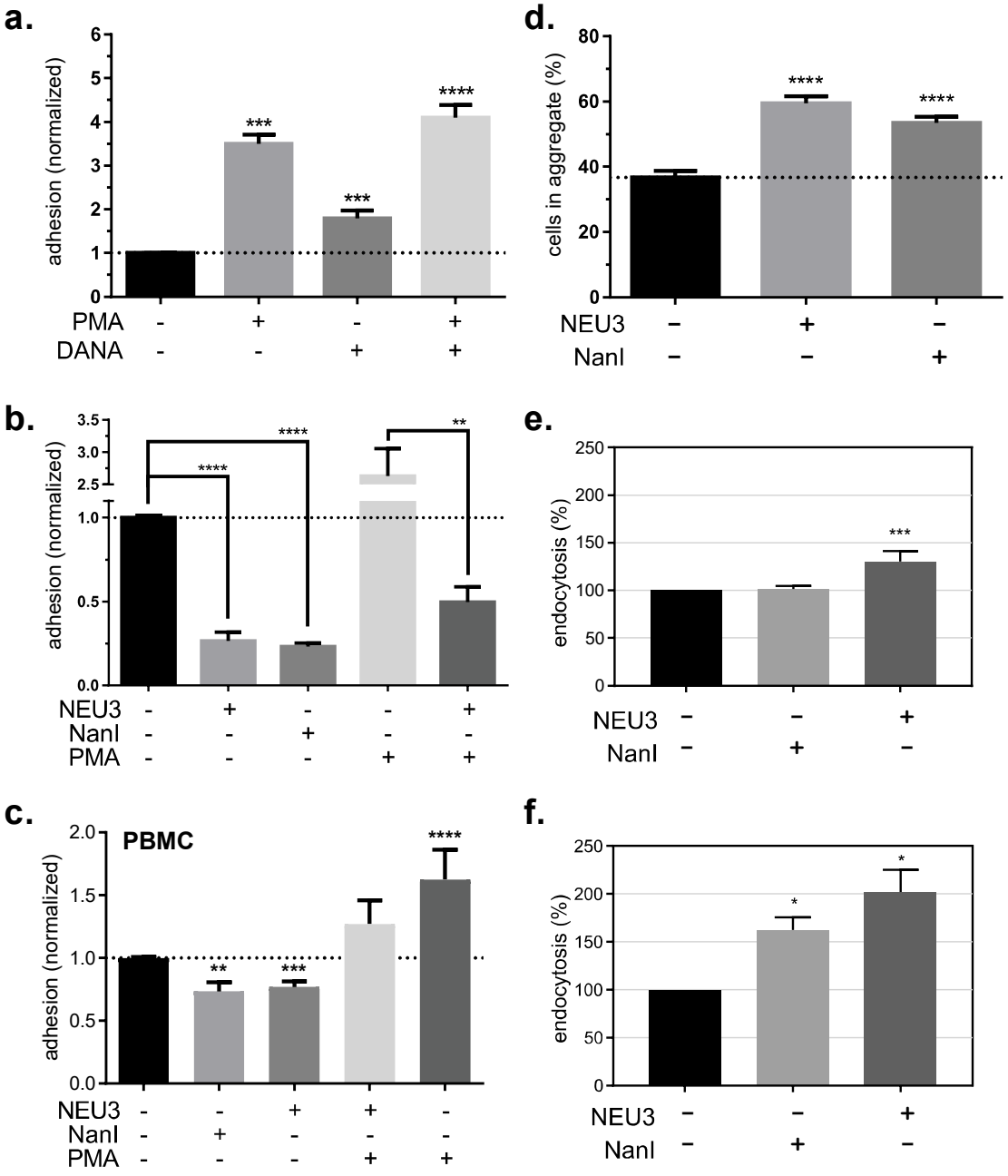

Figure 2.6. Adhesion of T cells is altered by Neu treatment. (**a.**) Adhesion of Jurkat cells to ICAM-1 was determined using flow cytometry and fluorescent beads (1 μm) under the indicated conditions. Control samples were treated with DMSO (0.05 %), PMA, or DANA for 30 min. (**b.**) Adhesion of Jurkat cells to ICAM-1 was determined using flow cytometry and fluorescent beads; samples were treated with buffer or enzyme for 3 h, followed by incubation with buffer,

PMA, or DANA (100 μM) for 30 min. All cytometry data were normalized to the appropriate control after background subtraction (BSA coated beads.) (**c.**) Isolated PBMC were treated as indicated and adhesion to ICAM-1 coated beads was determined by flow cytometry. Data shown are from at least three healthy donors for PBMC experiments. Values shown in a.-c. are the average of N = 6-12 replicates, error is the standard error of the mean. (**d.**) Homotypic aggregation of Jurkat cells was determined using microscopy. Cells were incubated under the indicated conditions for 3 h. Aggregation was determined by imaging to determine the total number of cells and the number of cells found within aggregates. Aggregation is expressed as the percentage of cells in all samples found within an aggregate (N = 24, from two separate experiments), and error is shown as the standard error of the mean. (**e.**) Changes in the endocytosis of β2-integrin (N = 4) and (**f.**) β1-integrin (N = 2) in Jurkat cells after treatment with NanI or NEU3 (30 min at 37 °C). Error bars are shown as standard error of the mean, all data were compared to the indicated control using a t-test; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.005$; ****, $p \leq 0.0001$.

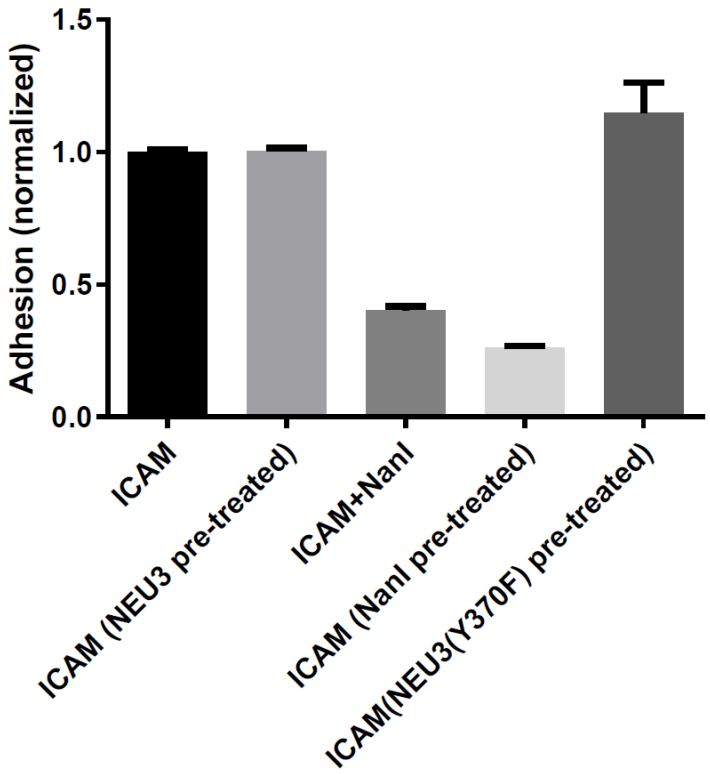

Figure 2.7: ICAM-1 adhesion controls. Adhesion of Jurkat cells to ICAM-1 was determined using flow cytometry and fluorescent beads (1 μm) under the indicated conditions. Beads were coated with ICAM-1 for all experiments shown. Where indicated, the ICAM-labelled beads were pre-treated with the enzyme indicated in parenthesis (3 hrs pH 7.2), washed and then used for adhesion experiments. For ICAM+NanI, the beads were co-incubated with the enzyme during the adhesion experiment. Cells were treated with ICAM beads under conditions identical to Figure 5. The NEU3(Y370) mutant was prepared as previously reported.[35] Error bars are shown for SEM.

2.2.6 NEU3 enhanced homotypic aggregation

Once we had concluded that NEU3 was a negative regulator of β2-integrin mediated adhesion, we investigated the effect of NEU3 on an alternative cell adhesion process – homotypic aggregation. The homotypic aggregation of T cells is generally considered to be mediated by multiple receptors[55-57] including LFA-1[58] and VLA-4[59, 60]. We determined the number of cells involved in aggregates using microscopy (**Figure 2.6d**). Cells were treated with NEU3 or NanI, both of which resulted in significantly increased aggregation. Previous results have found that NEU3 increased fibronectin–β1 integrin cell migration in epithelial cells, and the effect was not due to desialylation of fibronectin.[61] Homotypic aggregation of neutrophils has been reported to be increased by treatment with NanI.[62] We concluded that while NEU3 disrupted LFA-1–ICAM-1 interactions (*vide infra*), desialylation of cell surface targets by NEU3 or NanI also stimulated other adhesion mechanisms. These two results indicate that sialic acid can be either activating or inhibitory in adhesion, likely due to the target SGC involved.

A control experiment was conducted with cells incubated under the indicated conditions for 3 hrs. Aggregation was determined by imaging and analysis with CellProfiler (version 2.1.1) to determine the total number of cells and the number of cells found within aggregates. Inactive mutant of NEU3 was used to treat cells, which did not produced any difference in aggregate (**Fig 2.8**).

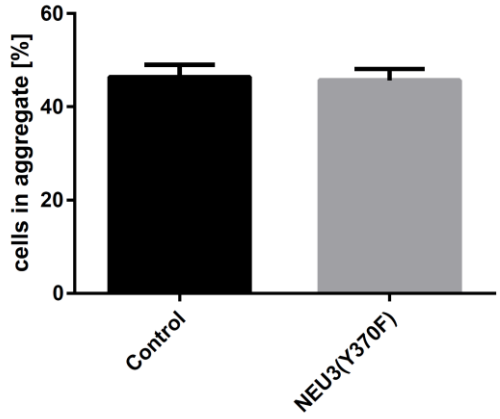

Figure 2.8: Homotypic aggregation controls. Homotypic aggregation of Jurkat cells was determined using microscopy. The NEU3(Y370) mutant was prepared as previously reported.[63] Aggregation is expressed as the percentage of cells in all samples found within an aggregate (N = 24, from two separate experiments), and error is shown as the standard error of the mean.

2.2.7 NEU3 altered endocytosis of β1 and β2 integrins

Previous studies have supported a role for glycolipids in the regulation of integrin endocytosis.[28] Our examination of the effect of NEU3 on integrin adhesion suggested differential regulation of these two adhesion receptors. Perturbation of the balance of exo- and endocytosis of integrins is well known as a mechanism to regulate adhesion.[64, 65] We used biotin labeling of cell-surface proteins to interrogate changes to endocytosis of β2 and β1 integrins in Jurkat after exposure to NEU enzymes. We observed a significant increase in endocytosis of the β2 integrin after NEU3 treatment, but NanI appeared to have no significant effect (**Figure 2.6e**). In contrast, the β1 integrin showed a large increase in endocytosis after both NEU3 and NanI treatment (**Figure 2.6f**). We note that these analyses are based on densitometry of multiple Western blots and are best interpreted qualitatively.

2.2.8 Neuraminidases altered expression of LFA-1 epitopes

Our observation that NEU3 activity blocked LFA-1 adhesion could be the result of multiple mechanisms. To gain some insight into the process, we measured changes in known surface epitopes of LFA-1. The MEM148 epitope is found in the membrane proximal domain of CD18, and is an activation-dependent epitope of LFA-1.[66] The TS1/22 antibody binds to the LFA-1 α-chain, and is both adhesion blocking and conformationally independent.[67] To detect changes in epitope expression after treatment with NEU, we used flow cytometry (**Figure 2.9**). Treatment of cells with NEU3 showed a significant increase in the MEM148 epitope on Jurkat, but not on PBMC. NanI treatment resulted in a decrease in the

MEM148 epitope on both cell types. The TS1/22 epitope showed a more dramatic increase in expression on both cell types after NEU3 treatment, while NanI had no detectable effect on this epitope. These data are consistent with increased LFA-1 total expression levels upon NEU3 treatment, with minor changes to the MEM148 activation epitope. Increased LFA-1 expression may be a result of delivery of LFA-1 to the surface from intracellular stores.[68] We note that previous reports have observed increased expression of the MEM148 epitope and increased surface-localized LFA-1 on neutrophils after NanI treatment.[13]

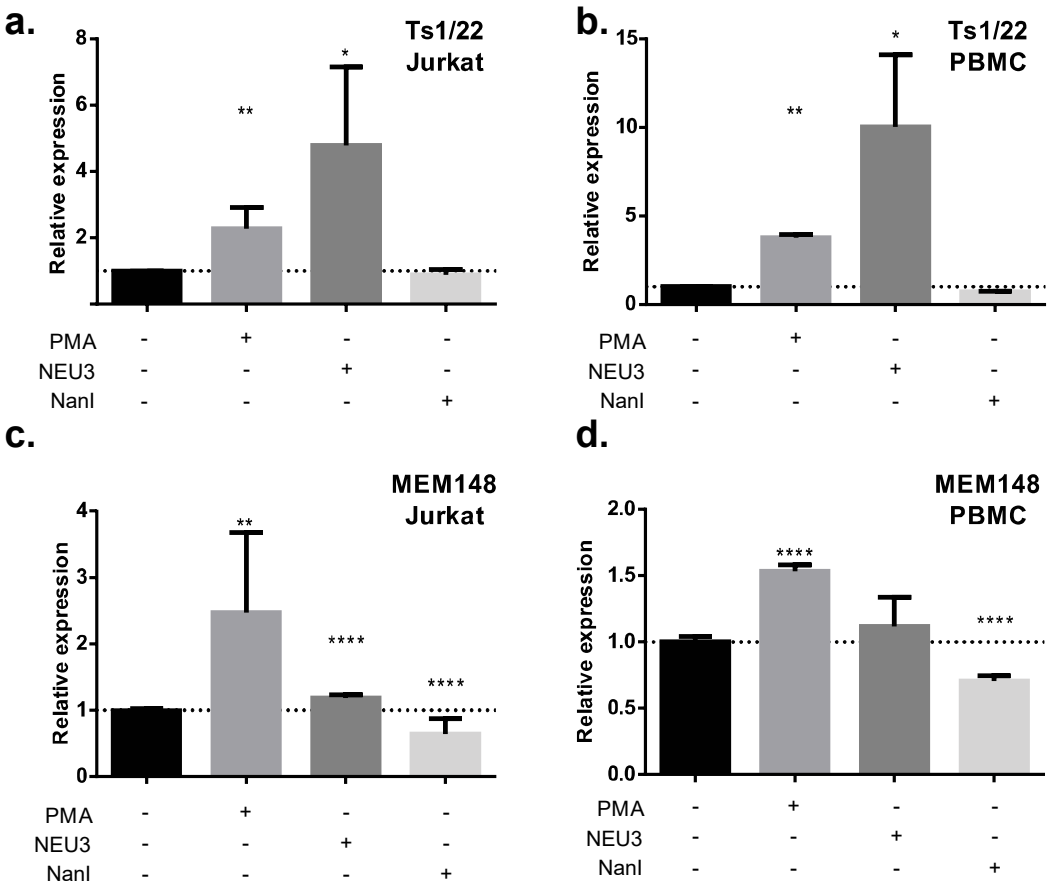

Figure 2.9. Alteration of LFA-1 epitopes by neuraminidase treatment. Jurkat T cells (**a.** and **c**.) or PBMC (**b.** and **d.**) were treated with the indicated conditions. Treated cells were labelled with primary antibodies (TS1/22 or MEM148), followed by an AF647-conjugated secondary antibody. Cells were fixed with 1% PFA, analyzed by flow cytometry, and normalized to control (buffer) treatment. Data shown are the mean of three replicates for each sample and error is shown as the standard error of mean. Data were compared to the appropriate control using a t-test to determine p values; *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.005$; ****, $p \leq 0.0001$.

2.3 **Discussion**

Our data demonstrated that the human NEU3 enzyme can act as a negative regulator of LFA-1–ICAM-1 mediated adhesion in lymphocytes. We confirmed that treatment of cells with exogenous NEU3 resulted in a reduction of sialylated glycolipids, such as GM3, and also modified the glycan of LFA-1. NEU3 has been previously shown to prefer glycolipid substrates over glycoproteins, due to its requirement for substrates with a hydrophobic aglycone.[26, 37] Treatment of cells with NEU3 or NanI (which lacks activity for gangliosides),[36] produced increased LFA-1 clustering by fluorescence microscopy. Quantitative analysis of single-cell images confirmed that LFA-1 clustering was increased in NEU-treated cells. Analysis of the lateral mobility of LFA-1 found that NEU3 treatment resulted in an increase in integrin diffusion. Importantly, substantial effects on lateral mobility were only observed after NEU3 treatment, supporting a critical role for gangliosides in regulating integrin diffusion.[28] NEU activity had a significant influence on LFA-1–ICAM-1 adhesion, and we concluded that NEU3 blocked LFA-1 adhesion through desialylation of a cell-surface target (e.g. glycolipids or glycoproteins). Control experiments confirmed that the NEU3 anti-adhesive effect was not a result of changes to ICAM-1 glycosylation, as was the case for NanI. Furthermore, we confirmed that NEU3 activity was distinct from NanI in that NEU3 induced an increase in surface expression of LFA-1 (**Figure 2.9**). We observed that NEU3 activity increased endocytosis of both β1 and β2 integrins while NanI only affected the β1 integrin, suggesting a role for glycolipids in

regulating the balance of exo- and endocytosis of adhesion receptors. Together, our results implicate NEU3 as a potential regulator of β2-integrin mediated adhesion.

LFA-1 activity is governed by the interplay of avidity and affinity regulation; and these factors correspond to receptor clustering and conformational change, respectively.[34, 69, 70] A quantitative analysis of single-cell images obtained by TIRF microscopy confirmed that a larger proportion of LFA-1 was found in clusters on NEU–treated cells. In accord with this observation, we found that the lateral mobility of LFA-1 on NEU–treated cells was increased, providing a mechanism for the change in integrin organization.[44] LFA-1 has been observed to cluster in microdomains[71] with tightly regulated lateral mobility.[72] The size of LFA-1 clusters has been estimated over a wide range between 50-200 nm.[30, 40, 73] Our observations using TIRF microscopy are limited by diffraction, and therefore changes observed for cluster size (**Figure 2.4**) were likely due to co-localization of multiple microclusters. The lateral mobility of LFA-1 has been found to be dependent on conformational state and stimulation of the cell.[45, 49] Furthermore, LFA-1 mobility is linked to the ability of the cell to form a stable adhesion.[51]

The two NEU enzymes studied here had distinct effects on LFA-1. We observed that NEU3 activity induced an LFA-1 activation epitope on Jurkat, but had no effect on PBMC.[66] Treatment of cells with NanI showed a uniform decrease in the MEM148 activation epitope. Glycosylation of integrins is known to influence both conformation and function,[74-76] and our lectin blots confirm that these enzymes modify glycosylation of LFA-1. While NanI activity also resulted in a blockade of LFA-1–ICAM-1 interactions, this effect can be ascribed to modification of the

ICAM-1 ligand, rather than targets on the lymphocyte. These observations may be specific to cell types, as NEU activity directed at LFA-1 is reported to enhance adhesion of neutrophils.[13] In addition to conformational changes of integrins, desialylation may alter inter-molecular interactions which depend on the revealed galactoside epitopes generated by NEU activity.[77, 78]

Signaling mechanisms known to negatively regulate LFA-1 include the protein tyrosine phosphatase receptor type γ,[79] and the Lyn kinase.[80, 81] Notably, Lyn activity suppresses LFA-1–ICAM-1 adhesion, but enhances cell migration. Lyn activity is known to be regulated by glycolipid composition of the outer leaflet.[82] Furthermore, Lyn is known to be found at the leading edge of migrating cells,[83] as is NEU3 and consistent with our imaging in **Figure 2.3**.[84] We note that Lyn-mediated activation of alternative adhesion mechanisms is consistent with our homotypic aggregation results (**Figure 2.6**). Future investigations will need to address the link between Lyn and NEU3 as it pertains to LFA-1 down-regulation. While our data suggest NEU3 is a negative regulator of LFA-1 adhesion, we also observed an increase in homotypic aggregation in NEU3-treated cells. Homotypic aggregation of lymphocytes is mediated by receptors including the αLβ2,[58] α4β1, and α5β1 integrins.[85] The activity of NEU3 on simple gangliosides (e.g. GM3) would generate neutral glycosphingolipids, which are known to be activators of homotypic aggregation in hematopoietic cells.[86] Furthermore, changes in membrane cholesterol or GM1 are known to disrupt LFA-1 adhesion, and our results may suggest that changes to other glycolipid components of microdomains have a similar effect.[41] Increased NEU3 activity may alter the concentration of

additional degradation products of GSL. Ceramide is known to increase surface expression of β2 integrin, and to block β2-integrin–dependent adhesion while preserving homotypic aggregation.[87]

How do NEU enzymes affect LFA-1 function? First, the mechanisms of action for each NEU enzyme used in our study show important differences. The substrate profile of each enzyme is different, with NanI acting on glycoproteins while NEU3 uses glycolipids as its favored substrates.[36, 88] Thus, NanI treatment is likely to alter glycoprotein substrates, while NEU3 modifies both glycolipids and LFA-1. Changes to glycoprotein epitopes of LFA-1 may block adhesive interactions[89] or induce engagement of new protein-glycan interactions.[90-92] Galectins are secreted lectins that bind to β-galactoside epitopes, which are often revealed by NEU activity.[93] Galectin-1 can inhibit leukocyte adhesion,[94, 95] while Galectin-3 can promote neutrophil adhesion.[96] Previous work has found that native NEU activity in neutrophils positively regulates LFA-1 adhesion, which may implicate other isoenzymes, such as NEU1, for this activity [13]. NEU3 activity altered lipid composition and could therefore influence membrane microdomain recruitment, function, or trafficking of integrins.[97-99] It is possible that direct integrin-glycolipid interactions are responsible for the reorganization of LFA-1 observed here, as β1-integrins are known to bind directly to gangliosides.[100] Indeed, changes in membrane glycolipid composition have been shown to affect the recruitment of integrins and Src kinases to membrane microdomains.[101] Ectoenzymes are emerging as important regulators of leukocyte migration.[102]

NEU3 has been established as a plasma membrane-associated enzyme,[20] and our work here confirms that its activity can negatively regulate leukocyte adhesion.

The data presented here provide evidence that the human NEU3 enzyme acts as a negative regulator of LFA-1–ICAM-1 adhesion. However, it is important to emphasize that the enzyme also activates other cell-adhesion mechanisms (**Figure 2.6d**). The enzyme alters glycolipid composition, which likely leads to a shift in clustering and increased endocytosis of LFA-1. This mechanism of LFA-1 regulation was able to substantially block PMA-activated adhesion of leukocytes, and may present a novel target for pharmacological intervention in inflammation.[54] Although blocking of NEU3 would result in positive regulation of LFA-1, inhibition may also block the downstream adhesion mechanisms which are activated by NEU3. Future work should address the specific adhesion mechanisms which NEU3 may positively regulate, and the role of NEU3 within the inflammatory cascade.

2.4 Experimental procedures

2.4.1 Cell culture

Jurkat cells (clone E6.1) were grown in RPMI 1640 medium with 10% fetal bovine serum (FBS) at 37 °C under 5% CO_2 to approximately 1.5 x 10^6 cells ml^{-1}. Phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich. Oakville, Ontario, Canada) and cytochalasin D (cytoD) (ENZO Life Sciences. Farmingdale, NY, USA) were dissolved in dimethyl sulfoxide (DMSO) as stock solutions (Sigma-Aldrich. Oakville, Ontario, Canada). Human neuraminidase 3 (NEU3) and NanI

(Sigma-Aldrich. Oakville, Ontario, Canada) were stored in the same NEU3 buffer (0.2 M NaCl, 10% glycerol, 10 mM maltose, 20 mM MOPS pH 7.2).[35]

Peripheral blood mononuclear cells (PBMC) were isolated from whole blood samples of healthy donors following a protocol approved by the Health Research Ethics Board of the University of Alberta (Pro00016491). Briefly, cells were centrifuged over a ficoll gradient, transferred to a culture flask, and incubated overnight at 37 °C with 5% CO_2 in RPMI medium (10% FBS and 1% penicillin-streptomycin).

Cell treatments were performed using the conditions indicated below. A suspension of 1 x 10^6 cells was taken from the culture flask and washed three times with buffer. In all washing steps, the cells were spun at 1200 rpm for 2 min in a desktop centrifuge. For PMA and cytoD conditions, cells were re-suspended in 1 mL of buffer (PBS) with DMSO (0.05% final concentration) or the same buffer with DMSO containing PMA (200 ng mL^{-1}) or cytoD (2.5 μg mL^{-1}). The samples were then incubated at 37 °C under 5% CO_2 for 30 min. For enzyme treatments, cells were re-suspended in PBS alone, NEU3 enzyme (0.01875 U), or NanI enzyme (0.01875 U). One unit of enzyme activity was defined as, 1 U = 1 μmol 4MU-NANA substrate cleavage min^{-1}, this calibration was done at pH 4.5. Enzyme samples were then incubated at 37 °C under 5% CO_2 for 3 h. After incubation all treated cells were then washed 3 times with PBS before further labelling, analysis, or extraction steps.

2.4.2 High-performance thin layer chromatography (HPTLC)

For high-performance thin layer chromatography (HPTLC) experiments, phosphate buffered saline (PBS) was used as washing buffer. All treatments were done with 1 x 10^7 cells in a 10 mL volume. After treatment, the cells were centrifuged to a pellet and re-suspended in 60 µL water, and sonicated for 30 sec. Cells were extracted with a mixture of chloroform and methanol (1:1, 400 µL x 3) and agitated for 10 min. The sample was centrifuged (10,000 rpm for 10 min), and the supernatant was transferred to a glass bottle, dried under a flow of N_2, and stored at -20 ºC. Before analysis, the cell extract was dissolved in a chloroform and methanol solution (1:1, 200 µL) and applied to a HPTLC plate (Sigma-Aldrich) using a glass micropipette. Chromatography was performed in with acetic acid, *n*-butanol, and 0.25% $CaCl_2$ (1:2:1) as the eluent followed by staining with orcinol (0.5 g orcinol, 200 mL 8% H_2SO_4 in ethanol).

2.4.3 Extraction and purification of gangliosides

Ganglioside extraction and purification was performed following previous reports.[32] Briefly, a lysate of 1 x 10^6 Jurkat T cells was diluted with ice cold water (4 mL g^{-1} based on weight of sample). After homogenization, methanol was added to make the final ratio of methanol:water 8:3. Chloroform was added after vigorous mixing of this suspension to make the chloroform:methanol:water mixture to the ratio 4:8:3 (v/v/v). This mixture was vortexed and centrifuged at 1500 RPM for 15 mins. The supernatant was carefully recovered, volume measured, then diluted with 0.173 volumes of water. After mixing, the suspension was centrifuged again. The upper phase was recovered and transferred to a fresh tube, purified on a SepPak

C18 cartridge (Waters Corporation, Milford, MA, USA), evaporated to dryness under a stream of nitrogen and re-dissolved in methanol.

2.4.4 LC-MS analysis of gangliosides

Expression and purification of EGCase was performed following previous reports with a pET30 vector.[33] Samples of extracted GSLs were dissolved in a 50 mM sodium acetate buffer (pH 5.2) containing 1 mg mL^{-1} sodium cholate and incubated for 18 h at 30 °C with 0.086U EGCase. One unit of EGCase I was defined as the amount of enzyme that hydrolyzes 1 μmol of GM3 per minute at 30 °C. Released glycans were labeled with a mixture containing 30 mg anthranilic acid, 20 mg boric acid, 40 mg sodium acetate, and 45 mg sodium cyanoborohydride at 80 °C for 45 min.

Labeled glycans were analyzed using an Agilent 1200 SL HPLC system and a normal-phase column (Accucore-150-Amide-HILIC, 2.6 μm, 2.1 x 150 mm, Thermo Fisher). Dried samples were re-solubilized in water:DMF:acetonitrile in the ratio 1:1:2 and 15 μL was injected. The fluorescence detector was set to monitor at 320 nm excitation and 420 nm emission, and all chromatography was performed at 40 °C. Mass spectra were acquired in negative mode using an Agilent 6220 Accurate-Mass TOF HPLC/MS system with a dual spray electrospray ionization source along with a secondary reference sprayer for a reference mass solution. Data analysis was performed using the Agilent MassHunter Qualitative Analysis software package version B.07.01.

2.4.5 Single-particle tracking of integrin receptors

In single particle tracking and TIRF imaging experiments the washing buffer was HBSSB (1% BSA, Hank's Balanced Salt Solution). TS2/4 mAb was purified from HB244 cell line (American Type Culture Collection, ATCC). A Cy5-antibody conjugate was generated using an NHS ester of Cy5 (GE Healthcare, Buckinghamshire, UK) using the manufacturer's protocol. The dye:antibody ratio was measured at 3.7 dye per antibody after purification. A final concentration of 30 ng mL^{-1} of labeled protein was added into a sample of 1 x 10^6 treated cells. Cells were washed 3 times with HBSSB buffer after labeling at 37 ºC for 15 min. Labelled cells were re-suspended in 1 mL HBSSB and transferred to a 24-well cell culture plate containing a coverglass which was previously treated with 10 µg mL^{-1} of poly-L-lysine. The plate was spun at 400 g for 7 min, and the well was washed 3 times with HBSSB to remove unattached cells. The coverglass was transferred onto a microscopy slide and sealed with Cytoseal 60 (Thermo Fisher Scientific, Waltham, MA). All tracking data were acquired within 30 min of sealing. Tracking videos were taken on a NIKON Ti TIRF microscopy with 638 nm laser excitation and a 690 ± 40 nm filter with a 60 x TIRF objective (NA 1.49) with an additional 1.5 x magnifier (providing a final resolution of 252 nm $pixel^{-1}$). Videos were acquired at 10 FPS for 10 sec and analyzed with u-track [48] in Matlab (2012b). Trajectories with fewer than 20 steps were discarded. The intensity of the trajectories was used to exclude the top and bottom 5 % of trajectories from the analysis. The data were processed using custom scripts in MATlab.[45]

2.4.6 Total internal reflection fluorescence microscope (TIRF) Imaging and cluster analysis

Cells were treated identically to those used for SPT, followed by fixation with 1% paraformaldehyde (PFA) in PBS at 4 ºC for 60 min. The fixed cells were washed with PBS twice, and 2 x 10^5 fixed cells from each treatment were re-suspended in 200 µL PBS. The cells were labelled with Cholera toxin B FITC (CTB-FITC, 5 µg mL^{-1} ; Sigma-Aldrich, Oakville, Ontario, Canada) and TS2/4-Cy5 (500 ng mL^{-1}) at room temperature for 10 min. Labelled cells were washed twice with PBS, re-suspended in 1 mL PBS, and transferred to a 24-well cell culture plate containing a coverglass (poly-L-Lysine treated). The plate was spun at 400 g for 7 min, and the well was washed 3 times with PBS. The coverglass was transferred to a microscope slide and sealed with Cytoseal 60, followed by imaging using TIRF. More than three independent labelling samples were imaged for each treatment.

TIRF imaging for cluster analysis was performed using an identical protocol as described above, with a lower concentration of the TS2/4-Cy5 conjugate (80 ng mL^{-1}). Fifteen cells were chosen for analysis based on DIC (the cells were apparently healthy and round) and staining (TIRF image showed TS2/4 labeling on the whole cell). Images of individual cells were processed in ImageJ by applying a threshold to identify labeled pixels and processed using the analyze particle function to measure clusters larger than 4 $pixel^2$.

2.4.7 ICAM-1 Adhesion assay

To prepare ICAM-1–bead complexes purified ICAM-1 protein (5 µg, R&D systems, Minneapolis, MN, USA) was incubated with 25 µL of a 2% solution of 1

μm microbeads (yellow-green sulfate microspheres; Life Technologies, Burlington, ON, Canada) in a final volume of 100 μL (in 50 mM PBS, pH 8.3) for 8 h at 4 ºC. After incubation, a solution of PBS (50 mM) containing 2% BSA (100 μL) was added and the suspension of beads and was incubated overnight at 4 ºC. The beads were stored at 4 ºC and used within 24 h.

Jurkat T cells or PBMC were treated as above with PBS as the washing buffer. Treatment with DANA was at 100 μM. Treated cell samples contained 3 x 10^6 cells in 1 mL PBS, and were labeled with 10 μL of ICAM-1 or control beads followed by incubation at 37 ºC for 15 min. The labeled cells were washed with PBS three times and resuspended in 1 mL of PBS followed by analysis on an Accuri C6 flow cytometer.

2.4.8 Homotypic aggregation of Jurkat cells

Jurkat cells were washed with PBS three times and re-suspended in PBS at a concentration of 2 x 10^5 cells mL^{-1}. Cells were transferred to solutions with the following conditions: buffer alone, NEU3 (0.01875 U), or NanI (0.01875 U). All samples had a final concentration of 10% NEU3 storage buffer and 0.6% binding buffer (100 mM CAPS, 0.15M NaCl, 1 mM calcium chloride, pH 11.0). All samples were stained with 1 μg mL^{-1} Calcein AM (Life Technologies, Burlington, ON, Canada). Samples were transferred to a 96-well- plate (200 μL per well). The plate was incubated at 37 ºC for 3 h. Fluorescent images were taken with a NIKON Ti microscope using a 20x objective and a FITC filter set. Four images were taken for each well to provide 24 images for each condition. The images were analyzed with CellProfiler (Version 2.1.1)[103, 104]. The total number of the cells in each image,

and the number of single cells (cells not in any clusters) were counted in CellProfiler. The amount of aggregation was calculated as (total number of cells – numbers of single cells)/total number of cells. Results were confirmed using at least two independent repeats.

2.4.9 Integrin endocytosis

Samples of sulfo-NHS-SS-Biotin and streptavidin-resin were obtained from Thermofisher, USA. Glutathione (GSH) was purchased from Sigma-Aldrich. Antibodies for β1 integrin (clone EP1041Y), β2 integrin (clone EP1286Y), HRP-conjugated goat anti-rabbit secondary antibody (ab6721) were obtained from Abcam, USA.

Biotin-based endocytosis assays were performed as previously described with slight modifications.[105] Jurkat T cells were grown in 10% FBS-containing medium T-75 flasks (Corning, USA). Cells were collected by centrifugation at 300 g for 2 min and 2 x 10^6 cells were placed in separate Eppendorf tubes. Samples were placed on ice and washed once with cold PBS, and then labeled with 0.8 mg mL^{-1} of sulfo-NHS-SS-biotin for 60 min at 4 °C. Cells were then centrifuged again and unbound biotin was washed away with cold medium. Cells were then resuspended in prewarmed medium with or without treatment and biotin-labelled surface proteins were allowed to internalize at 37 °C for 30 min. Enzyme treatments were performed in PBS buffer (pH 7.0) with 0.02 U of NEU3 or NanI. Cold medium was immediately added, and samples were put over ice. Any remaining biotin at the cell surface was removed with GSH buffer (75 mM sodium chloride, 1 mM magnesium chloride, 0.1 mM calcium chloride, 50 mM GSH, and 80 mM

sodium hydroxide) for 30 min at 4 °C, followed by multiple washes with cold PBS. The cells were pelleted and treated with lysis buffer (150 mM sodium chloride, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0 and phosphatase and protease inhibitor cocktails (Roche, USA)) at 4 °C for 30 min. The lysate was clarified by ultra-centrifugation at 18,000 x g for 10 min. Supernatants were collected, and BCA assay was used to calibrate protein concentrations. Equal amounts of protein were incubated with streptavidin-resin with agitation at 4 °C overnight. The resin was washed once with lysis buffer and boiled with 2x Laemmeli sample buffer containing 100 mM DTT. Endocytosed biotinylated β1 and β2 integrins were measured by separate western blots for the respective β-chains.

2.4.10 LFA-1 Antibody binding

MEM148 antibody was purchased from AbD Serotec (Raleigh, NC, USA); TS1/22 antibody was purchased from Fisher Scientific Ottawa, ON, Canada. A sample of cells (Jurkat or PBMC, 1 x 10^6) were treated with NEU3 (0.01875 U) or NanI (0.01875 U) for 3 h, or PMA (200 ng mL^{-1}) for 30 min. Incubations were done at 37 °C at 5% CO_2, followed by washing with PBS, centrifugation, and re-suspension in PBS buffer (900 μL). Cells were then divided into aliquots and labeled with TS1/22 or MEM148 antibodies for 30 min. Cells were then washed three times with PBS and re-suspended with PBS with AF-647–conjugated secondary antibody at 1:1000 dilution for 10 min. Cells were again washed three times and fixed with 1 % PFA for 10 min before analyzing using an Accuri C6 flow cytometer.

2.4.11 Lectin blotting of LFA-1

Purified LFA-1 (R&D systems, USA; 50 μg) was biotinylated with sulfo-NHS-SS-biotin and immobilized on Neutravidin resin (600 μL) overnight at pH 7. The suspension was then washed three times with PBS buffer (pH 7.0). The immobilized LFA-1 was then treated with NEU3 (0.01875 U) or NanI (0.01875 U) and the mixture was incubated for 3 h at 37 °C. The resin was washed three times to remove contaminating proteins, followed by incubation at 95 °C for 10 min in the presence of DTT (100 mM) to release LFA-1 from the resin. Equal amounts of the protein were then loaded on an SDS-PAGE gel and detected using biotinylated peanut agglutinin (PNA) or *Sambucus nigra* (SNA) lectins (Bio-World, Ohio, USA) at 1:500 dilution. Lectins were imaged with streptavidin-HRP (1:200 dilution).

2.5 **References**

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3. Isoenzyme-Selective Inhibitors of Human Neuraminidases Reveal Distinct Effects on Cell Migration [1]

[1] Compounds used in these studies were synthesized and characterized by Yi Zhang and Tianlin Guo; NEU1-HEK cells were provided by Alexey V. Pshezhetsky (University de Montreal); A version of this manuscript has been submitted for publication in ACS Chemical Biology.

3.1 **Introduction**

Human neuraminidase enzymes (hNEU) are glycosyl hydrolases which release sialic acids from glycolipid and glycoprotein substrates.[1] Currently, hNEU isoenzymes that have been identified include NEU1,[2] NEU2,[3] NEU3,[4] and NEU4.[5] Though the four human neuraminidase isoenzymes catalyze the same hydrolysis reaction of sialoglycoconjugates (SGC), their specific targets and expression allow them to play differing roles in human physiology. One process that illustrates the diversity of hNEU function is the migration of cells.[6, 7] NEU1 has been reported to downregulate cell migration in human lung cancer cells and to suppress metastasis in mice.[6, 8] NEU1 also regulates lysosomal exocytosis, which may influence the invasiveness of cancers.[9] In contrast, NEU2 has been linked to increased cell survival and motility of prostate cancer cells.[10] Increased expression of NEU3 has been reported in human colon,[11] melanoma,[12] glioblastoma,[13] and prostate cancer cells[14] where the enzyme affects cell motility.[6] Additionally, NEU3 activity is implicated in axon regeneration.[15] NEU4 is overexpressed in human neuroblastoma resulting in increased cell proliferation,[16] but it is downregulated in human colon cancer cells where expression correlates with increased motility and metastasis.[17] These findings suggest significant, but highly diverse, roles played by hNEU enzymes in cell migration and cancer metastasis.

Integrins are glycoprotein receptors that play central roles in cellular migration, metastasis, and transmigration.[18, 19] Fibronectin (FN) is a major extracellular matrix (ECM) ligand of the α4β1 and α5β1 integrins (also called VLA-4 and VLA-5, respectively)[20] commonly expressed on many cell types.[21, 22] Integrins are regulated

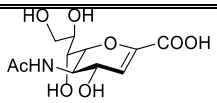
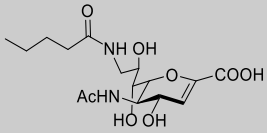
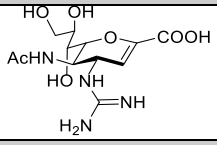
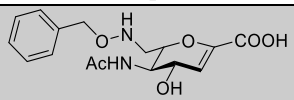
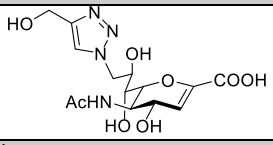
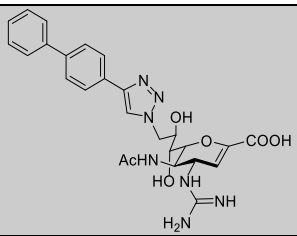
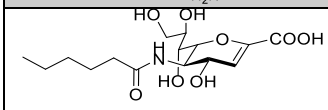
in part by dynamic shuttling of receptors between the membrane and endocytic compartments by clathrin-dependent and clathrin-independent mechanisms.[23-26] The endo- and exocytosis of integrins is known to feature selective uptake and spatially-organized processes;[27] furthermore, integrin cluster size and organization is critical to the strength of the resulting adhesions.[28] The glycosylation of β1 integrins plays an important role in integrin endocytosis and function.[29] The N-glycan structure of α5β1 influences endocytosis of the receptor through interactions with Galectin-3.[30-32] The N-glycan of the α5 integrin is critical to cell migration and may affect endocytosis.[33] Integrin-galectin interactions are attenuated by increased α2,6-linked sialic acid content, which masks lectin binding sites.[34, 35] Thus, hNEU may participate in integrin regulation by altering integrin-lectin binding.[36] Indeed, NEU1 is known to modify the N-glycan of the β4 integrin.[8]

The expression of multiple hNEU isoenzymes in most cell types makes it challenging to study the role of individual enzymes in cell migration. One strategy that has emerged is the use of selective small-molecule inhibitors of individual isoenzymes. We and other groups have worked to identify inhibitors of hNEU with excellent selectivity and nanomolar potencies, a selection of these are provided in **Table 3.1**.[37] Commonly used inhibitors include DANA **1** (2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid), a pan-selective inhibitor of the four hNEU isoenzymes with minor preference for NEU3 and NEU4.[38] The first compound designed as a selective inhibitor of hNEU was C9-BA-DANA **2**,[39] which targets NEU1. The antiviral and bacterial NEU inhibitor, zanamivir **3**, has low micromolar activity against NEU2 and NEU3.[40, 41] The first NEU3-selective inhibitor reported was

compound **4**.[38] The most potent inhibitor reported for any hNEU is the NEU4-selective inhibitor, C9-4HMT-DANA **5**.[42] Recently reported inhibitors for NEU3 and NEU1, C9-4BPT-C4-G-DANA **6** and C5-HA-DANA **7**,[43, 44] provide the most potent methods to target these isoenzymes. We considered that the availability of this panel of inhibitors would allow us to analyze specific contributions of individual hNEU isoenzymes to cell migration.

In the current study, we examined the role of hNEU isoenzymes in the migration of four different human cancer cell lines from prostate (PC-3), breast (MDA-MB-231), lung (A549), and cervical (HeLa) tissue. We hypothesized that this panel of different cell lines could reveal the range of activity for hNEU isoenzymes found in disparate tissues. Furthermore, we wanted to use a panel of inhibitors that could interrogate multiple isoenzymes, including NEU1, NEU3, and NEU4. We observed that, not only do these cell lines show different levels of response to the same inhibitor, but that targeting of specific isoenzymes can have contrary effects. These results suggest that interrogating hNEU isoenzyme activity with pan-selective inhibitors could mask important activities and make a strong case for the further development and application of selective inhibitors of this family of human enzymes.

Table 3.1: Structures and activity of hNEU inhibitors used in this study.

Structure	Identifiers	IC_{50} [μM][f]				Selectivity [g]
		NEU 1	NEU 2	NEU 3	NEU 4	
	DANA[38] **1**	80	90	6	13	NEU3/4 (7X)
	C9-BA-DANA[a, 39] **2**	10	>500	>500	>500	NEU1 (50X)
	zanamivir[41] **3**	>500	8	4	47	NEU2/3 (6X)
	**4**[b, 38]	>500	>500	24	>500	NEU3 (21X)
	C9-4HMT-DANA[c, 42] **5**	>500	>500	80	0.16	NEU4 (500X)
	C9-4BPT-C4-G-DANA [d,43] **6**	>500	6	0.58	6	NEU3 (10X)
	C5-HA-DANA [e,44] **7**	0.42	15	210	440	NEU1 (36X)

a, Referred to as compound "10h" in original citation.[39] This compound has been named as a C9-butyl-*N*-amide derivative of DANA (C9-BA-DANA).[45] This could more appropriately be named as a C9-pentylamide, but we maintain this nomenclature for consistency with the literature.

b, Referred to as compound 5c in original citation.[38]

c, Referred to as compound 6 in original citation.[42] Named here as the C9-(4-hydroxymethyltriazolyl)-DANA.

d, Referred to as compound 8b in original citation.[43] Named here as the C9-(4-biphenyltriazolyl)-C4-guanidino-DANA.

e, Referred to as compound 11d in original citation.[44] Named here as the C5-hexylamido-DANA.

f, IC_{50} values from previous studies against a 4MU-NANA substrate are provided with the indicated citation.

g, 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. For inhibitors with similar activity against more than one target, both targets are listed, and the selectivity given is based on the lowest potency.

3.2 **Results**

3.2.1 NEU4 suppress cell migration of PC-3 and MDA-MB-231 cells

To understand the specific functions of hNEU isoenzymes on cell migration, we investigated β1 integrin-mediated cell migration of four human cell lines (PC-3, MDA-MB-231, A549, HeLa). HeLa and A549 cells express α5β1 integrin, with low levels of α4β1.[36, 46] PC3 cells are known to express β1 integrins.[47] Glycosylation of the α5β1 integrin is critical for cell migration in MBA-MD-231 cells.[48] To restrict our findings to the β1 integrin, we used a FN-coated surface for cell migration assays, following previously reported methods.[31, 49] A panel of reported hNEU inhibitors with isoenzyme selectivity were either synthesized as reported or obtained from commercial sources (**Table 3.1**). To screen the activity of these compounds in each cell line, we tested their activity to alter cell migration at doses which were approximately ten times that of the lowest reported IC_{50} value of each compound. In these experiments, cytochalasin D (CytoD), an actin depolymerizing agent,[50] was used as a positive control, and showed significant reduction of migration across all cell lines (**Figure 3.1**, **Table 3.2**).

Table 3. 2. Normalized cell migration of HeLa, A549, PC-3 and MDA-MB-231 cells treated with hNEU inhibitors.

	HeLa		A549		PC-3		MDA-MB-231	
	Mean ± SEM	(N)	Mean ± SEM	(N)	Mean ± SEM	(N)	Mean ± SEM	(N)
control (Buffer)	100 ± 2	(38)	100 ± 2	(35)	100 ± 2	(37)	100 ± 2	(29)
CytoD (197 nM)	38 ± 2****	(37)	36 ± 3****	(34)	37 ± 2****	(37)	24 ± 1****	(30)
1 (100 µM)	99 ± 2	(32)	90 ± 2*	(25)	82 ± 2****	(27)	91 ± 2	(16)
2 (100 µM)	-		-		167 ± 8****	(6)	163 ± 10***	(5)
3 (10 µM)	95 ± 3	(26)	102 ± 2	(29)	78 ± 3****	(20)	63 ± 4****	(11)
4 (250 µM)	94 ± 3	(22)	93 ± 3	(23)	61 ± 2****	(14)	79 ± 3****	(16)
5 (1.5 µM)	90 ± 3*	(23)	96 ± 2	(15)	90 ± 3*	(14)	74 ± 2****	(23)

Migration is expressed as mean ± standard error of the mean (SEM) of the percentage of distance traveled compared to control. Values were compared to control using a Dunnett's t-test (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.005$; ****, $p \leq 0.0001$).

We first examined HeLa and A549 cell lines using a set of inhibitors with mixed activity as well as those that target NEU3 (compounds **1**, **3**-**5**). Several inhibitors tested in HeLa did not show significant reduction of cell migration, with only compound **5** having significant reduction (94 ± 3 %; **Figure 3.1A**). We previously found that both **5** and DANA **1** were active in a similar assay with HeLa cells; however, we note that the time of incubation for the current experiment is substantially longer here (3 h vs 21 h), which likely explains the discrepancy.[36] Testing in A549 cells found that DANA **1** was a moderate inhibitor of migration (90 ± 2%; **Figure 3.1B**), while the other compounds tested had no effect. These

results suggested that different cell lines show differential responses to the same compounds, and we proceeded to test our inhibitor panel on additional lines.

Testing in PC-3 cells identified multiple compounds as active inhibitors of cell migration (**Figure 3.1C**). The pan-selective inhibitor, DANA **1**, was active at 100 μM (82 ± 2%) and testing at a lower concentration (1.5 μM) did not show significant activity. Zanamivir **3** was an active inhibitor at 10 μM (78 ± 3%), but showed no effect at 1.5 μM. The NEU3-selective inhibitor, **4**, showed substantial inhibition at 250 μM (61 ± 2%), but had no effect at 1.5 μM. The NEU4-selective inhibitor **5** was active at 1.5 μM (90 ± 3%). MDA-MB-231 cells showed some similarities in sensitivity to hNEU inhibitors observed for PC-3 cells (**Figure 3.1D**). DANA **1** was not active over the range of 1.5 to 100 μM; while zanamivir **3** was only active at 10 μM (63 ± 4%). The NEU3-selective inhibitor, **4**, was only active at the higher concentration tested (250 μM; 79 ± 3%); while the NEU4-selective inhibitor, **5**, was active at 1.5 μM (74 ± 2%).

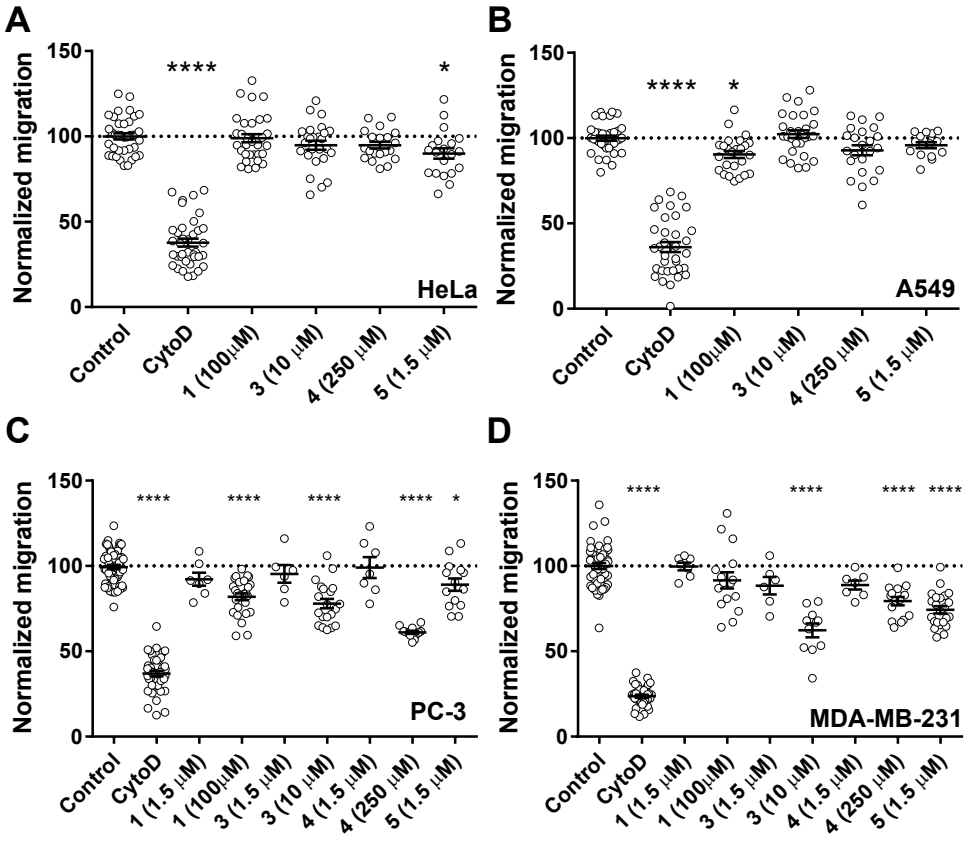


Figure 3.1. Inhibition of cell migration using inhibitors of neuraminidase enzymes. Normalized cell migration of four different cells treated with specific isoform-selective or non-selective inhibitors of human neuraminidase enzymes. A) HeLa, B) A549, C) PC-3, and D) MDA-MB-231. The data is presented as mean ± SEM, and conditions were compared to control using one-way ANOVA and Dunnett's t-test (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.005$; ****, $p \leq 0.0001$).

These initial migration data confirmed that inhibition of individual isoenzymes gave variability in the responses of each cell line. Furthermore, these data suggested that compounds with activity for NEU3 and NEU4 were active as inhibitors of cell migration in PC-3 and MDA-MB-231 cells. The HeLa and A549 cells were less sensitive to all compounds tested. We therefore focused our attention on understanding the role of hNEU isoenzymes in PC-3 and MDA-MB-231 cells.

3.2.2 Inhibition of NEU1 activates cell migration

After identifying PC-3 and MDA-MB-231 cells as sensitive to hNEU inhibitors, we proceeded to investigate the effects of inhibitors for NEU1 and NEU4. We used C9-BA-DANA **2**, a compound selective for the human NEU1 enzyme,[39] and C9-4HMT-DANA **5,** which was identified as a potent and selective inhibitor for NEU4.[42] These two compounds offered the best selectivity among available inhibitors, and we hypothesized that they would be most likely to show significant effects. We tested the dose-dependent effect of C9-BA-DANA **2** in PC-3 and MDA-MB-231 cells (**Figure 3.2A & 3.2B**). We were surprised to observe that treatment of both cell lines with compound **2** at 10-100 μM caused a significant *activation* of cell migration (up to 167 ± 9%, PC-3; 163 ± 10%, MDA-MB-231, **Table 3.3**). This was in contrast to the effects of other compounds tested above, which had no effect or else acted as inhibitors of migration. Testing of compound **5**, a selective inhibitor of NEU4, over a similar range found *inhibition* of cell migration between 1.5 – 100 μM (**Figure 3.2C & 3.2D**). Together, these experiments confirm that targeted inhibition of individual hNEU isoenzymes can have opposite effects in cells.

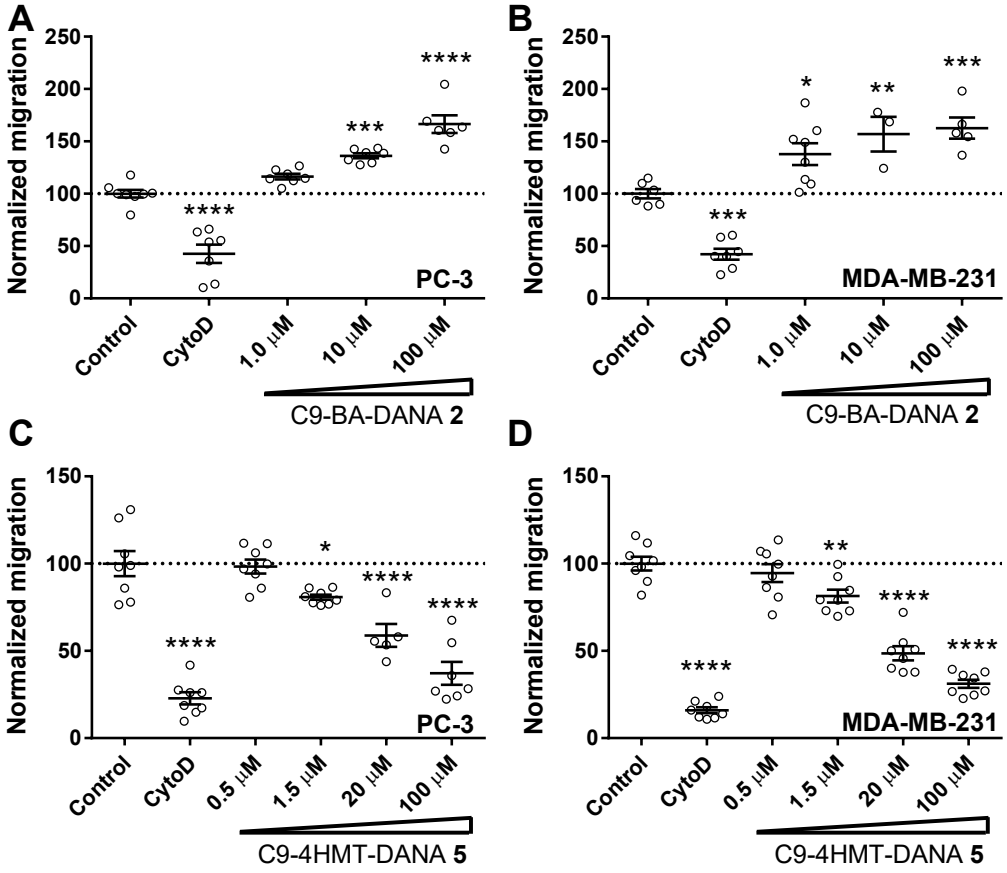


Figure 3.2. Dose-dependent cell migration modulation by hNEU inhibitors. Cell lines A) PC-3 and B) MDA-MB-231 were treated with 1.0, 10, and 100 μM C9-BA-DANA (**2)** and cell migration was determined. Cell lines C) PC-3 and D) MDA-MB-231 were treated with 0.5, 1.5, 20, and 100 μM C9-4HMT-DANA (**5)** and cell migration was determined. Cytochalasin D (197 nM) was used as a positive control. The data is presented as mean ± SEM, and conditions were compared to control using one-way ANOVA and Dunnett's t-test (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.005$; ****, $p \leq 0.0001$).

Table 3.3. Normalized cell migration of PC-3 and MDA-MB-231 cells treated with hNEU inhibitors 2 & 5.

	PC-3		MDA-MB-231	
	Mean ± SEM	(N)	Mean ± SEM	(N)
Control (Buffer)	100 ± 4	(8)	100 ± 5	(6)
CytoD (197 nM)	43 ± 9****	(7)	42 ± 5***	(7)
2 (100 µM)	167 ± 9****	(7)	163 ± 10***	(8)
2 (10 µM)	136 ± 3****	(7)	157 ± 17**	(3)
2 (1 µM)	116 ± 3	(6)	138 ± 10*	(5)

	PC-3		MDA-MB-231	
	Mean ± SEM	(N)	Mean ± SEM	(N)
Control (Buffer)	100 ± 7	(8)	100 ± 4	(8)
CytoD (197 nM)	23 ± 4****	(8)	16 ± 2****	(8)
5 (100 µM)	37 ± 7****	(8)	31 ± 2****	(8)
5 (20 µM)	59 ± 7****	(8)	49 ± 4****	(8)
5 (1.5 µM)	81 ± 1*	(5)	81 ± 4**	(8)
5 (0.5 µM)	98 ± 1	(7)	95 ± 5	(8)

Migration is expressed as mean ± standard error of the mean (SEM) of the percentage of distance traveled compared to control. Values were compared to control using a Dunnett's t-test (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.005$; ****, $p \leq 0.0001$).

To investigate the role of NEU1 in cell migration using a different method, we employed a previously reported human embryonic kidney (HEK-293) model which overexpressed NEU1.[51] We confirmed that NEU1 expression levels in these cells were elevated (approximately 5-fold relative to control cells) using western blotting (**Figure 3.3**). Determination of basal cell migration confirmed that increased NEU1 expression resulted in a large *decrease* in cell migration (62 ± 4 %) compared to untransfected HEK-293 cells (100 ± 2 %; **Figure 3.3**). This result is consistent with our finding that competitive inhibition of NEU1 resulted in *increased* migration.

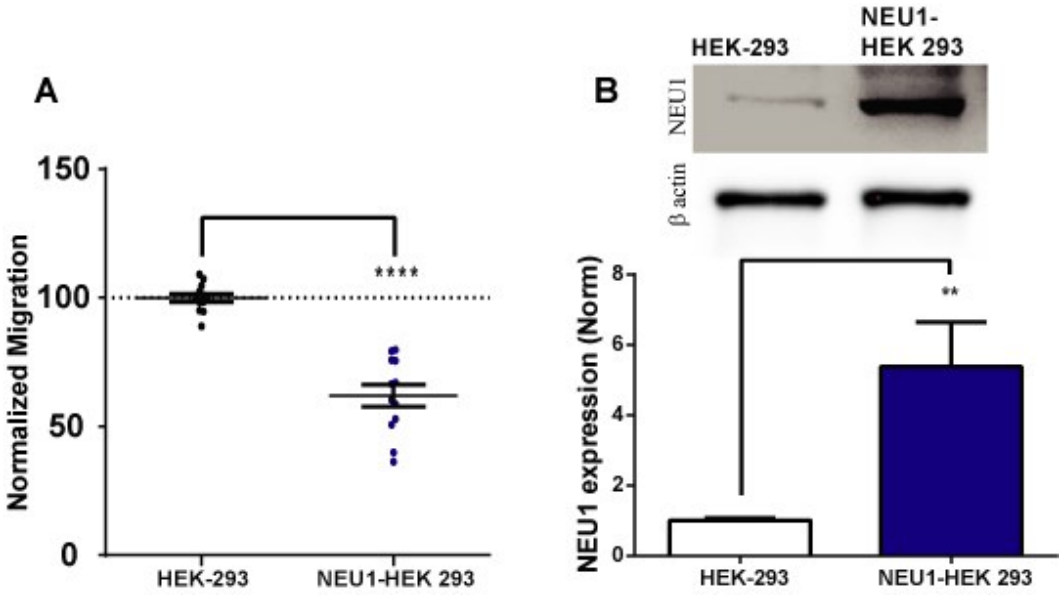


Figure 3.3. Effect of NEU1 overexpression on HEK 293 cell migration. The NEU1 isoenzyme was overexpressed in HEK293 cell lines using a previously described method.[51] A) Overexpression of NEU1 caused a significant decrease in the migration of HEK293 cell lines. B) Expression levels of NEU1 in control and transfected HEK293 cell lines were tested using western blotting. A single representative image from at least six blots for NEU1 is shown (top). Data from all blots are shown in the bar graph. The amount of enzyme was corrected to a β-actin loading control and normalized to control cells. Data are plotted as the mean ± SEM and compared to control using a student's t-test (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.005$; ****, $p \leq 0.0001$).

We next sought to understand if the action of hNEU inhibitors with opposing effects on migration could interfere with each other. Our initial results with the pan-selective inhibitor, DANA **1** (**Figure 3.1**), where the inhibitor had no detectable effect in some cases, could be explained through mixed effects on NEU1 versus other isoenzymes. Thus, we tested the action of compound **2** on cell migration in the presence of NEU3 and NEU4 inhibitors (**Figure 3.4, Table 3.4**). Compound **4**, a NEU3-selective inhibitor, partially blocked the increased cell migration observed for **2** alone in both cell lines (125 ± 4%, PC-3; 131 ± 7%, MDA-MB-231). The NEU4-selective inhibitor, **5**, was able to block the activating effect of **2**, reducing the effect in both cell lines (103 ± 6%, PC-3; 102 ± 6%, MDA-MB-231). Combination of NEU1, NEU3, and NEU4 inhibitors returned cell migration to control levels (**2**+**4**+**5**, 103 ± 5%, PC-3; 111 ± 9%, MDA-MB-231). Finally, we observed that compounds **4** and **5** together had reduced effect on cell migration relative to each compound alone (104 ± 2%, PC-3; 116 ± 5%, MDA-MB-231). These experiments confirm that inhibitors of individual hNEU isoenzymes can have competing effects on cell migration and highlight the advantages of highly-selective inhibitors.

Table 3.4. Cell migration of simultaneous inhibition of multiple hNEU enzymes in the PC-3 and MDA-MB-231 cells.

	PC-3		MDA-MB-231	
	Mean ± SEM	(N)	Mean ± SEM	(N)
control (Buffer)	100 ± 4	(7)	100 ± 8	(8)
CytoD (197 nM)	30 ± 3****	(5)	47 ± 9****	(4)
2 (10 µM)	144 ± 4****	(21)	161 ± 8****	(12)
2 + 4 (100 µM + 250 µM)	125 ± 8*	(7)	131 ± 7*	(6)
2 + 5 (100 µM + 1.50 µM)	103 ± 6	(7)	102 ± 6	(6)
4+ 5 (250 µM + 1.5 µM)	104 ± 2	(6)	116 ± 5	(6)
2 + 4 + 5 (100 µM + 250 µM + 1.50 µM)	103 ± 5	(7)	111 ± 9	(5)

Migration is expressed as mean ± standard error of the mean (SEM) of the percentage of distance traveled compared to control. Values were compared to control using a Dunnett's t-test (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.005$; ****, $p \leq 0.0001$).

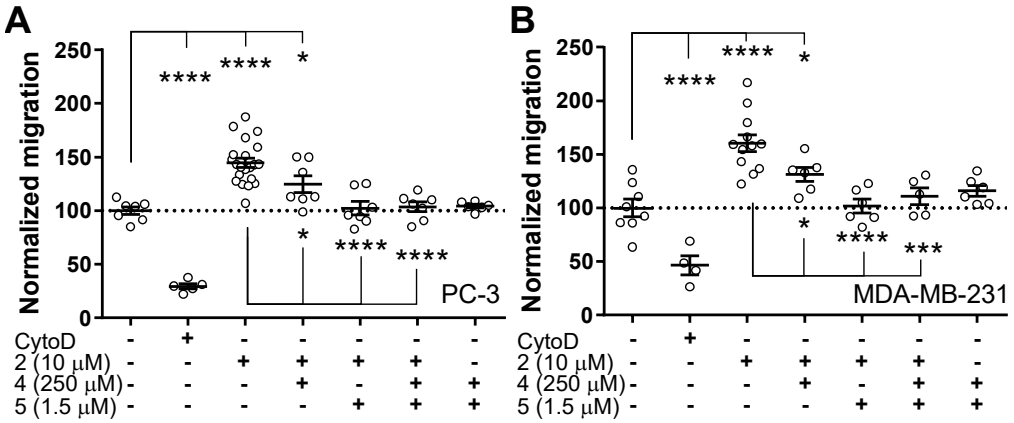


Figure 3.4. Combined effects of NEU inhibition. Normalized cell migration of four different cell lines treated specific NEU1 inhibitors alone or in combination with specific inhibitors of NEU3 and NEU4 enzymes in A) PC-3, and B) MDA-MB-231 cell lines. The data is presented as mean ± SEM, and conditions were compared to control using one-way ANOVA and Dunnett's t-test (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.005$; ****, $p \leq 0.0001$).

It is possible that reduced cell migration observed upon inhibitor treatment was due to toxicity of compounds to cells. To test this hypothesis, we evaluated the cytotoxicity of compounds **1**-**5** in PC-3 and MDA-MB-231 cells. Compounds were tested under identical conditions used in cell migration experiments, with the highest concentration used for each compound tested. We did not observe any significant cytotoxicity for most hNEU inhibitors, with the notable exception of C9-BA-DANA **2** (**Figure 3.5**, **Table 3.5**). Treatment of cells with the NEU1-selective C9-BA-DANA **2** at 100 μM resulted in significant reduction in cell viability in both cell lines (PC-3, 71 ± 3%; MDA-MB-231, 74 ± 2%). CytoD induced marginal toxicity for MDA-MB-231 cells only (90 ± 2%). Since C9-BA-

DANA **2** treatment of cells resulted in increased migration, we conclude that the toxicity of the compound was moderate, and was unlikely interfere with the migration assay. Overall, these results rule out compound toxicity as an explanation for reduced cell migration.

Table 3.5. Cytotoxicity of hNEU inhibitors.

	PC-3		**MDA-MB-231**	
	Mean ± SEM	(N)	Mean ± SEM	(N)
control (Buffer)	1.00 ± 0.02	(54)	1.00 ± 0.01	(72)
1 (100 µM)	1.01 ± 0.03	(32)	1.03 ± 0.02	(32)
cyto D (197 nM)	0.97 ± 0.02	(16)	0.90 ± 0.02	(32)
2 (100 µM)	0.71 ± 0.03****	(48)	0.74 ± 0.02****	(32)
3 (10 µM)	0.94 ± 0.03	(32)	1.05 ± 0.02	(32)
4 (250 µM)	1.02 ± 0.03	(36)	0.99 ± 0.02	(58)
5 (1.5 µM)	1.00 ± 0.03	(15)	0.97 ± 0.01	(32)

Migration is expressed as mean ± standard error of the mean (SEM) of the percentage of distance traveled compared to control. Values were compared to control using a Dunnett's t-test (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.005$; ****, $p \leq 0.0001$).

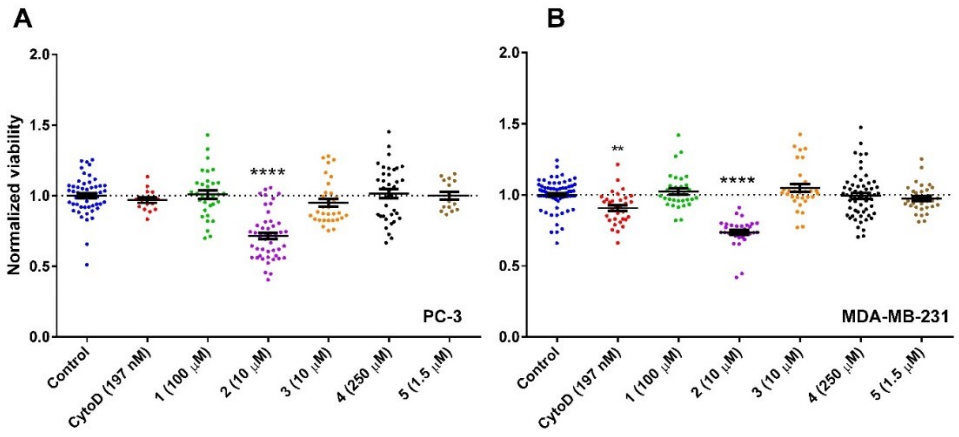


Figure 3.5. Toxicity profile of the PC-3 and MDA-MB-231 cells with inhibitors. A) PC-3 cells. B) MDA-MB-231 cells were treated with the highest concentrations of inhibitors used for migration studies and assessed for viability. For each cell line, 2.5 x 10^4 cells were incubated in each well of a clear 96 plate in the presence of inhibitors for 18 h. After incubation, 20 µL of MTS solution was added to each well and incubated for 1 hour. The absorbance of soluble formazan product was measured at 490 nm using a plate reader (Molecular devices) and viability of the cells were calculated. Data are plotted as the mean ± SEM and compared to control using a Dunnett's t-test (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.005$; ****, $p \leq 0.0001$).

3.2.3 Inhibition of cell migration is dependent on hNEU isoenzyme expression

The extent of effects for hNEU inhibitors varied among the four cell lines tested (**Figure 3.1**). To better understand these differences, we investigated the native expression of NEU1 and NEU3 proteins in each cell line using Western blotting (**Figure 3.6**). Expression levels were quantified by densitometry of western blots, and normalized to PC-3 cells (the lowest expressor). The cell lines which were least sensitive to NEU3 inhibitors, HeLa and A549, had the highest expression of the enzyme (6- and 14-fold relative to PC-3). The cell lines which were most sensitive to NEU3-selective compounds (PC-3 and MDA-MB-231) had the lowest expression levels among the cells tested. These findings suggest that cells with lower expression of hNEU are more sensitive to the action of hNEU inhibitors.

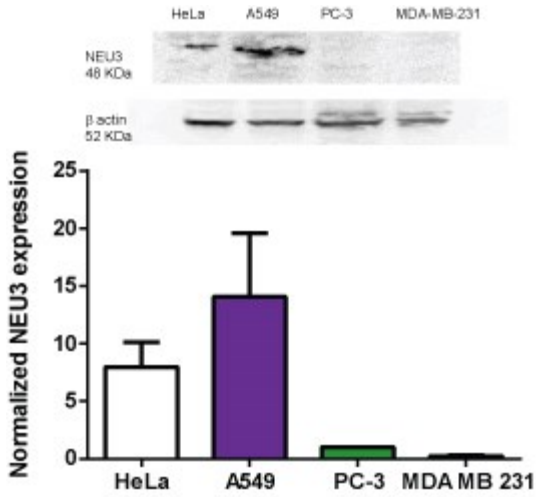


Figure 3.6. Differential expression levels of the NEU3 enzyme. NEU3 expression levels in HeLa, A549, PC-3, and MDA-MB-231 cell lines were determined using western blotting using mouse anti-human monoclonal antibody (MBL international, clone 11B). An image from a single representative blot is shown for NEU3 (top). At least six experiments were performed for cell line and the results are summarized in the lower bar graph. The amount of enzyme expression was corrected to a β-actin loading control and normalized to that of PC-3 cells and plotted as mean ± SEM. Data are plotted as the mean ± SEM.

3.2.4 Exogenous glycolipids modulate cell migration

To investigate the mechanism of action of hNEU inhibitors on cell migration, we tested the role of exogenous glycolipids on cell migration. Glycolipids such as GM3 and LacCer are known to affect integrin-mediated cell migration in HeLa and A549 cells,[36] and the concentration of these glycolipids could be altered by the activity of NEU3 or NEU4 enzymes.[4, 52] We treated cells with exogenous GM3 (50 ng mL^{-1}) and LacCer (50 ng mL^{-1}), in the presence of serum, to favor cell membrane insertion (**Figure 3.7, Table 3.6**).[53] We found that the addition of GM3 caused a significant decrease in cell migration of PC-3 cells (73 ± 7 %), but there was no measurable effect on MDA-MB-231 cells. However, in both cell lines LacCer treatment increased migration (130 ± 7%, PC-3; 150 ± 11%, MDA-MB-231). LacCer is the expected hydrolysis product of GM3 from NEU3, and has previously been reported to alter β1 integrin membrane organization, endocytosis, and cell migration.[54]

Table 3.6. Glycolipid effects on cell migration.

	PC-3		MDA-MB-231	
	Mean ± SEM	(N)	Mean ± SEM	(N)
Control (Buffer)	100 ± 5	(15)	100 ± 2	(12)
CytoD (197 nM)	32 ± 4	(14)	68 ± 2*	(16)
GM3 (50 ng/mL)	73 ± 7*	(16)	124 ± 16	(13)
Lac-Cer (50 ng/mL)	130 ± 7*	(14)	150 ± 11**	(14)

Migration is expressed as mean ± standard error of the mean (SEM) of the percentage of distance traveled compared to control. Values were compared to control using a Dunnett's t-test (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.005$; ****, $p \leq 0.0001$).

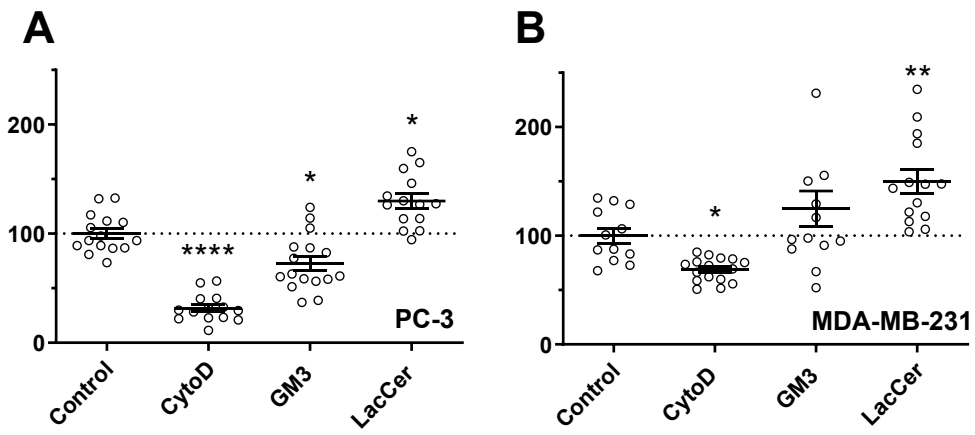


Figure 3.7. Effect of glycolipids on cell migration. Normalized migration of cells treated with exogenous glycolipids GM3 (50 ng/mL) and lactosylceramide (50 ng/mL) in A) PC-3 and B) MDA-MB-231 are shown. The data is presented as mean ± SEM, and conditions were compared to control using one-way ANOVA and Dunnett's t-test (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.005$; ****, $p \leq 0.0001$).

3.2.5 Inhibitors of hNEU cause changes in cellular GM2

The effect of hNEU inhibition is likely due to changes in specific SGC (e.g. glycolipids or glycoproteins) on cells which modulate integrin activity. NEU3 and NEU4 are implicated by our results in regulation of integrin-mediated cell migration, and these enzymes are known to modify glycolipid targets.[7, 52] We implemented an LC-MS based technique to quantify changes in glycolipids from PC3 cells, which were most sensitive to inhibitors, after treatment with hNEU inhibitors.[55-57] We quantified glycolipid concentrations for several glycolipid species on cells treated with inhibitors (**Figure 3.8**).

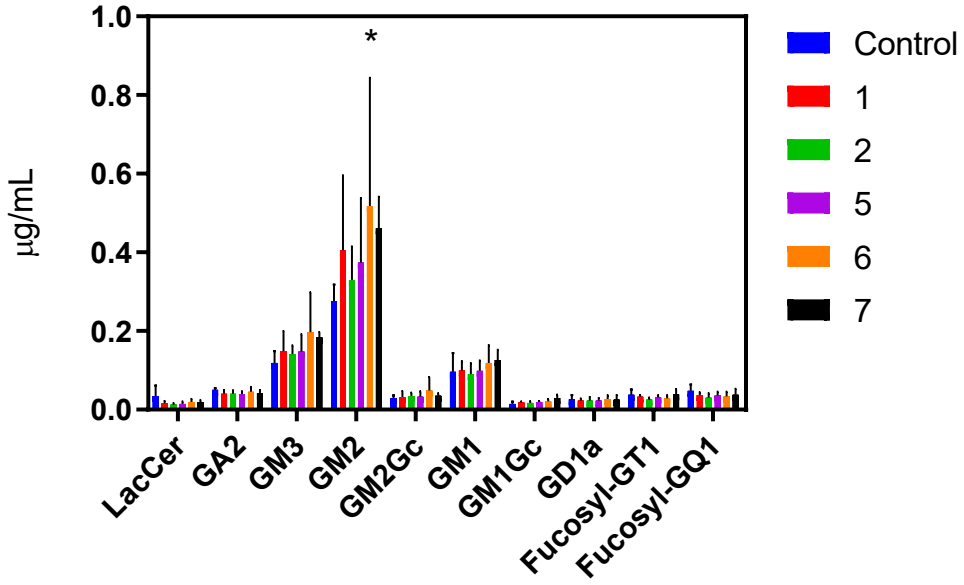


Figure 3.8. Glycolipid analysis of PC-3 cells treated with specific inhibitors. Cultured PC-3 cells were treated with neuraminidase inhibitors 1, 2, 5, 6, and 7 for 21 h. After that cells were harvested, lysed, and glycolipids were purified using protocol described in experimental methods. Gangliosides were treated with EGCase, followed fluorescent labeling of released oligosaccharides with 2AA. The labeled glycans were then analyzed by LC-MS (Agilent 6220 Accurate-Mass TOF HPLC/MS) and assigned using their corresponding mass by Agilent MassHunter Qualitative software. Data were compared to the amounts of each glycan present in control group using the student's t-test and plotted as the mean ± SEM (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.005$; ****, $p \leq 0.0001$).

In general, the three most abundant glycolipids were GM2, GM3, and GM1. There was significant variability in the levels of GM2 and GM3 detected for inhibitor-treated cells. A modest increase in the level of GM2 was observed after treatment with NEU3 inhibitor **6**, however other conditions did not show statistically significant changes (**Figure 3.9**). We concluded that, while glycolipid levels may be affected by inhibitor treatment, these changes were not detectable in our assay. A possible explanation for this observation is that the effects of inhibitors treatment may be transient and could be obscured over the long incubation times used to observe effects on cell migration.

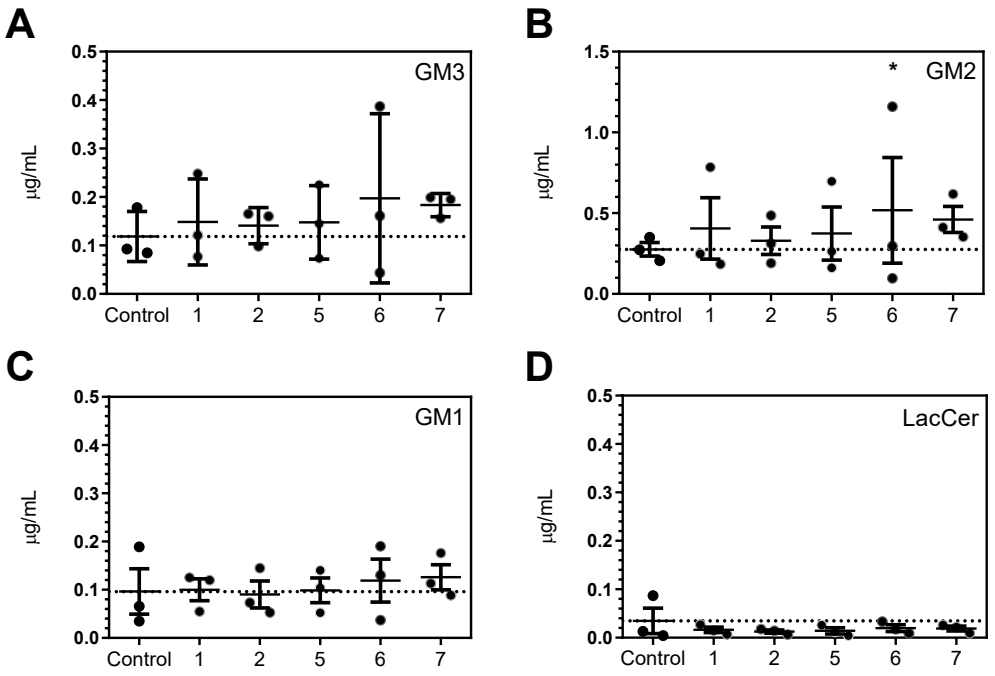


Figure 3.9. Changes in glycolipids of treated PC-3 cells. Glycolipid concentrations of PC-3 cells treated with selective inhibitors of hNEU at 100 µM concentration. A) GM3, B) GM2, C) GM1, and D) LacCer levels detected in PC-3 cells treated with compounds 1, 2, 5, 6, and 7 for 21 h. Data are presented as mean ± SEM, and were compared to control using one-way ANOVA and Dunnett's t-test (*, $p \leq 0.05$).

3.2.6 Cell surface sialoglycoconjugates are modulated by treatment with hNEU inhibitors

To explore the effects of inhibitors on cellular SGC, we examined changes to gross sialic acid content under these conditions. We quantified total sialic acid content by using fluorescent-conjugated lectins and flow cytometry. Cells were labeled with MAL (*Maackia amurensis* lectin), PNA (*Arachis hypogaea* lectin, peanut agglutinin), or SNA (*Sambucus nigra* agglutinin) (**Figure 3.10**). Cells were initially treated with buffer, **1** (100 μM), or NanI (0.1 RU). We observed significant changes in binding for MAL and PNA when both cell lines were treated with NanI. The binding of MAL (specific for sialic acid) was reduced, while PNA binding (specific for terminal galactose residues) was increased.[18] SNA (which prefers Neu5Ac α2-6 Gal terminal sites) was only reduced on PC-3 cells after NanI treatment. Although these findings were consistent with removal of sialic acids from the cell surface by NanI, treatment of cells with DANA **1** at 100 μM was unable to significantly alter the binding of MAL, PNA, or SNA to cells. High doses of DANA **1** (1 mM) were able to increase MAL and PNA staining in MDA-MB-231 (**Figure 3.11**). Furthermore, high dose treatment of MDA-MB-231 cells with zanamivir **3** (100 μM) resulted in an increase of MAL staining. These data confirmed that both **1** and **3** were able to affect gross sialic acid content of cells. However, we concluded that these changes may be small or limited to specific targets under the conditions used for cell migration assays, as they were not detectable by lectin staining at these lower concentrations.

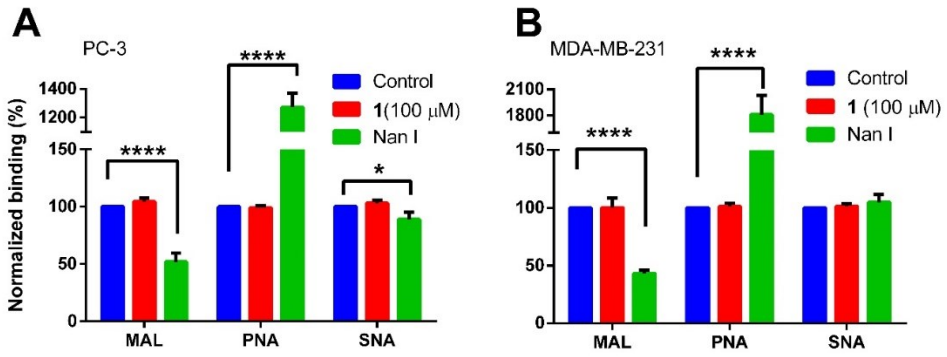


Figure 3.10. Changes in cell surface sialic acid content by lectin staining. A) PC-3 or B) MDA-MB-231 cells were treated with buffer or DANA (100 μM, 18 h). Cells were then detached (10 mM EDTA for 2 h in PBS) and harvested. For NanI treatment, cells were treated with 0.1 RU NanI for 2 h after harvesting. 1 x 10^5 cells were then stained with Peanut agglutinin (PNA, conjugated with Alexa Fluor 647, 20 μg/mL) or with Sambucus nigra agglutinin (SNA, conjugated with FITC, 20 μg/mL) or with Maackia amurensis lectin (MAL, conjugated with FITC, 5 μg/mL) for 30 mins separately. Fluorescence signal from MAL, PNA, and SNA staining was detected using flow cytometry, and normalized to the control group. Data are plotted as the mean ± SEM and compared to control using a student's t-test (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.005$; ****, $p \leq 0.0001$).

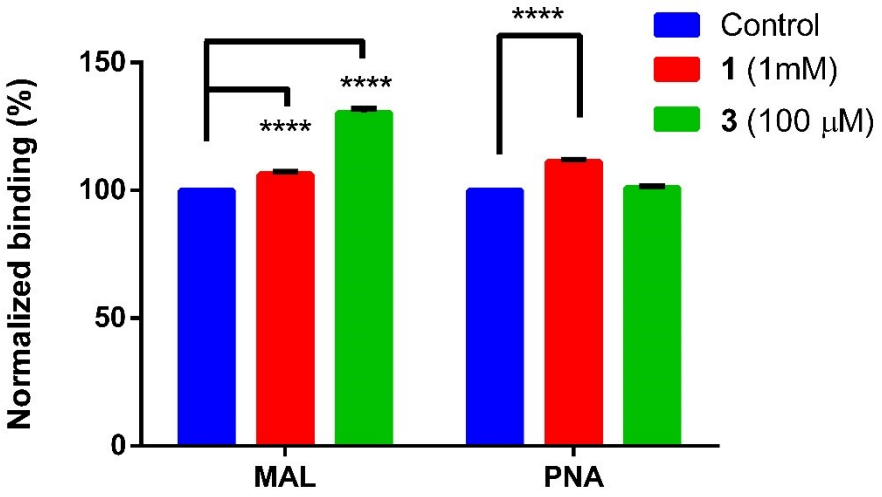


Figure 3.11. Changes in cell surface sialic acid content of MDA-MB-231 cells at high inhibitor concentrations. MDA-MB-231 cells were treated with DANA 1 (1 mM) and zanamivir 3 (100 mM) for 18 h. Cells were then detached (10 mM EDTA for 2 h in PBS) and harvested. For NanI treatment, cells were treated with 0.1 RU NanI for 2 h after harvesting. 1 x 10^5 cells were then stained with Peanut agglutinin (PNA, conjugated with Alexa Fluor 647, 20 μg/mL) or with Sambucus nigra agglutinin (SNA, conjugated with FITC, 20 μg/mL) or with Maackia amurensis lectin (MAL, conjugated with FITC, 5 μg/mL) for 30 mins separately. Fluorescence signal from MAL, PNA, and SNA staining was detected using flow cytometry, and normalized to the control group. Data are plotted as the mean ± SEM and compared to control using a student's t-test (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.005$; ****, $p \leq 0.0001$).

3.2.7 Human neuraminidases modulate glycosylation and endocytosis of β1 integrin

Our results confirmed that hNEU inhibitor treatment could result in changes to cellular SGC, including glycolipids and glycoproteins, which may affect integrin activity. Thus, we next tested for direct effects of hNEU on β1 integrin glycosylation and its endocytosis. The β1 integrin heterodimer is known to be heavily N-link glycosylated, and its glycosylation state may affect activity.[29, 31, 35, 48] To obtain evidence of changes to β1 integrin glycosylation, we performed western blotting of the β1 chain of the integrin heterodimer after treatment with NanI, NEU3, and NEU4 in MDA-MB-231 cells. This cell was selected because it previously showed sensitivity towards hNEU inhibitors (**Figure 3.12**).

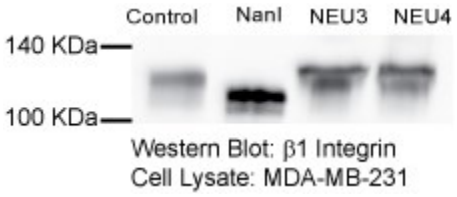


Figure 3.12. Molecular weight of the β1 integrin after neuraminidase treatment. MDA-MB-231 cells were treated with NanI or Neu3 for 3 h; and NEU4 for 1 h. Cells were lysed and the molecular weight of the β1 integrin was determined using a western blot using rabbit anti-integrin beta 1 antibody (EP1041Y) from Abcam, USA at 1:2000 dilution. Two separate experiments were performed and a representative image is shown.

We observed that the β1 integrin had a large decrease in molecular weight when treated with NanI, and a second smaller band appeared after treatment with NEU3 or NEU4. These data are consistent with a change in the integrin glycoform due to hNEU enzymes, most likely a loss of sialic acid consistent with previous experiments using β2 integrins.[57] To test whether NEU activity altered endocytosis of the integrin, we measured changes in the relative rate of β1-integrin endocytosis in PC-3 and MDA-MB-231 cells. Endocytosis was determined through biotinylation of cell surface proteins, and analysis of the distribution of the biotinylated proteins using western blotting.[58, 59] Conditions included the addition of exogenous enzymes, glycolipids, and hNEU inhibitors (**Figure 3.13**).

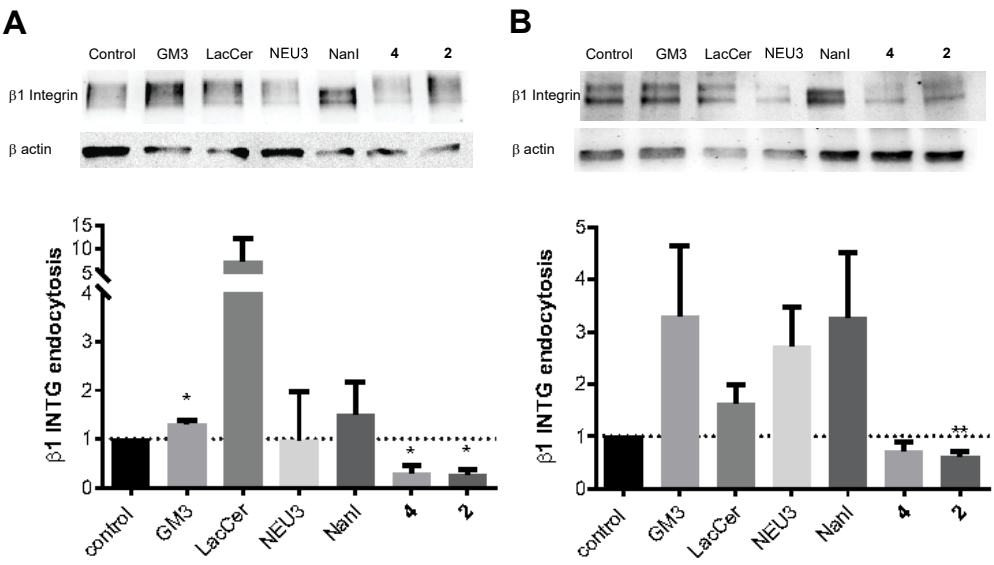


Figure 3.13. Neuraminidase enzymes affect the endocytosis of β1 integrin. The endocytosis of the β1 integrin was determined using biotinylation and western blotting in A) PC-3 and B) MDA-MB-231 cells. Blots are shown in the top rows for the β1 integrin, and bottom rows for β actin loading controls. Cells were treated with GM3 (50 ng/mL), LacCer (50 ng/mL), NEU3 (500 μRU), NanI (0.1 RU), 4 (250 mM), and 2 (100 μM) for 0.5 h and proceeded for blotting protocol. A representative blot from at least two experiments are shown here. Densitometry was used to compare changes in the integrin band to the loading control, and values are plotted as the mean ± SEM and conditions were compared to control using student's t-test (*, $p \leq 0.05$; **, $p \leq 0.01$).

Treatment with GM3 resulted in increased integrin endocytosis for both cell lines. LacCer treatment resulted in a large increase in endocytosis for MDA-MB-231 cells, and only a minor increase in PC3 cells. Exogenous NEU3 had no effect in MDA-MB-231 cells but resulted in an increase in endocytosis for PC3 cells. The bacterial neuraminidase, NanI, showed increased endocytosis for both cell lines. We note that NanI prefers glycoprotein targets[60] and may therefore implicate non-glycolipid SGC targets in this process. Treatment with NEU3 and NEU1 inhibitors (compounds **4** and **2**, respectively) reduced endocytosis of the integrin. Inspection of the integrin blots from these experiments provides further confirmation of changes to the glycoform of the integrin, as lower molecular weight bands can be observed in several conditions. Together, these results support a role for enzymes which modify SGC in the regulation of integrin endocytosis.

3.3 **Discussion**

Using migration assays and a unique panel of specific hNEU inhibitors, we have investigated the involvement of hNEU in regulation of integrin-mediated cell migration. Testing for general responses to inhibitors found different magnitudes of response for the same inhibitors among a sample of four human cell lines (PC-3, MDA-MD-231, A549, and HeLa). These differences were attributable in part to differences in hNEU expression levels. The response to inhibitors was dose-dependent, with NEU3 and NEU4 inhibitors blocking cell migration to various degrees. In contrast, treatment of cells with the NEU1 inhibitor, C9-BA-DANA **2**, had an activating effect on cell migration; while overexpression of the NEU1 enzyme in cells resulted in a corresponding decrease in basal cell migration. The

effects of isoenzyme-selective inhibitors were competitive with each other, as co-administration of NEU3 or NEU4 inhibitors with NEU1 inhibitors produced no net effect on cell migration. Experiments with exogenous glycolipids suggested a role for GSL in regulation of β1 integrin-mediated migration on FN, further supported by our finding of changes to cellular GM2 levels upon administration of inhibitors. These observations do not exclude a role for changes to other SGC, such as glycoproteins or the integrin itself. Lectin-staining of cells confirmed changes to cellular SGC after hNEU inhibitor treatment, which likely include both glycolipids and glycoproteins. Western blotting of the β1 integrin after exogenous NEU treatment confirmed that NEU3 and NEU4 induced changes in the molecular weight of the protein, consistent with desialylation. Finally, we evaluated changes to β1 integrin endocytosis on cells treated with exogenous NEU enzymes and hNEU inhibitors. These experiments revealed that NEU treatment generally increased endocytosis while inhibitors of hNEU blocked this process.

The modification of cellular SGC, including glycolipids and glycoproteins, contribute to the regulation of β1 integrin-mediated cell migration. We observed that exogenous GM3 inhibited migration of PC-3 cells consistent with previous findings.[36, 61] Treatment of cells with LacCer increased cell migration in both PC-3 and MDA-MB-231 cells. LacCer is known to activate β1 integrin clustering through activation of Src,[54] and to enhance endocytosis of β1 integrin[54] by a clathrin-independent mechanism.[62] Notably, NEU3 has been found to be associated with caveolin-1 and is found within membrane ruffles.[63, 64] Disruption of NEU3 function is expected to result in changes to GSL levels in cells. NEU3 knock-outs

show increased levels of LacCer, and, when combined with a HexA knock-out, result in large increases in GM2 levels.[65] NEU3 shRNA resulted in increased GM2 levels in neuronal cells.[66] This may be the result of increased levels of GM3 due to the loss of NEU3 activity; as knock-outs of GM3 synthase lead to reduced GM2 levels.[67] GM2 has direct molecular interactions with the β1 integrin,[68] and likely regulates integrin function in tumor cells through CD82.[69] Colon cancer cells show increased expression of NEU3, and a concomitant increase in LacCer concentrations.[11] LacCer and other GSL are internalized via caveolin-related mechanisms.[62] Our analysis of changes to cellular glycolipids confirmed that certain inhibitors of hNEU increased GM2 levels, and we propose that this is the most likely GSL involved in modulating β1 integrin function in cell migration. We note that our findings do not exclude a role for changes to integrin glycosylation in regulating cell migration. We find evidence of desialylation of β1 integrin after NEU treatment, consistent with previous studies.[8, 29, 57]

Our finding that selective inhibitors of hNEU can have disparate cellular effects provides an important caveat for future studies.[37] First, we note that testing of non-selective inhibitors, such as DANA **1**, can provide misleading results. In our case, testing with only DANA **1** would have led us to erroneously conclude that hNEU inhibitors only block cell migration. The same may also be true for commercially available viral inhibitors such as zanamivir **3**, which has mixed effects on NEU2 and NEU3.[40, 41] Studies that exploit isoenzyme-selective hNEU inhibitors are becoming more common;[36, 45, 70, 71] however, identifying new, more selective, and

more potent inhibitors remains an active area of investigation.[37, 38, 42-44] These compounds may provide new avenues for therapeutics.[37]

The regulation of cell migration by hNEU, and the availability of small molecule inhibitors for these enzymes, suggest potential applications. Blocking of integrin-mediated cell migration has been explored in the development of anti-adhesive strategies in cancer.[72, 73] Thus, understanding the specific roles of hNEU activity in regulating cell migration will be essential to testing the feasibility of inhibitors as therapeutics. The enzymatic activity of NEU1 has been investigated for effects on various cellular adhesion and migration receptors. The regulation of β1 integrin is critical for wound healing.[74] Increased NEU1 expression blocks endothelial cell migration, but NEU3 had only a minor effect on this process.[75] Increased NEU1 activity reduced invasive liver metastasis in animal models.[8] The activity of NEU1 has been shown to activate CD44 binding to hylauronin.[76] In endothelial cells increased NEU1 activity leads to desialylation of CD31, and reduced cellular adhesion.[77] Increased NEU1 expression has also been shown to decrease epithelial cell migration.[78] Sialidase activity accelerates fibrosis, and can be activated by cytokines in A549 and PBMC.[79] We observed that NEU1 overexpression reduced integrin-mediated cell migration on FN, consistent with previous findings that NEU1 overexpression reduced in vivo metastasis of tumor cells.[8]

The contrasting roles of NEU1 and NEU3/NEU4 in the regulation of cell migration suggest that selective targeting could allow for applications in wound healing.[74, 80] While anti-adhesive strategies are generally concerned with blocking cell migration, wound healing applications may benefit from strategies to activate

cell migration. Our findings suggest that enzyme activity of NEU3/NEU4 activates cell migration, while NEU1 inhibits cell migration. Correspondingly, inhibitors of NEU3/NEU4 will block cell migration and inhibitors of NEU1 will activate cell migration.[79] Therefore, strategies to enhance NEU3/NEU4 activity or to block NEU1 should lead to increased β1 integrin-mediated cell migration.

3.4 Conclusion

In this study, we have shown that β1 integrin-mediated cell migration is differentially regulated by individual hNEU isoenzymes. Selective inhibitors for hNEU isoenzymes revealed that NEU1 inhibition was activating, while NEU3 and NEU4 inhibition attenuated cell migration. These effects were competitive, and we confirmed that inhibitors with mixed activity, or mixtures of selective inhibitors, could mask effects on cell migration. These results highlight the need for selective tools to target hNEU, and the fact that isoenzymes can play distinct roles in regulating cellular processes. Finally, the importance of integrin receptors to cancer metastasis and wound healing suggest that hNEU inhibitors may provide a new target for pharmaceutical strategies.

3.5 Materials and Methods

3.5.1 Cell culture

HeLa cells were a generous gift of Prof. Robert Campbell, (University of Alberta, Canada). MDA-MB-231 cell line was a generous gift of Prof. Ratmir Derda (University of Alberta, Canada). PC-3 cells were a generous gift of Prof. Jeremy Wolf (University of Victoria, Canada). A549 cells were obtained from

ATCC. All cell lines were cultured according to ATCC guidelines. Briefly, HeLa and MDA-MB-231 cell line were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin in 5% CO_2 and 37 °C in a humidified incubator. PC-3 and A549 were cultured in Ham's F-12k medium supplemented by 10% FBS and 1% penicillin-streptomycin in 5% CO_2 at 37 °C incubator. All experiments were performed with cell of passage 5 to passage 15.

3.5.2 Sources of reagents

DANA **1**, **2**, **4**, **5**, **6**, and **7** were prepared as previously reported.[38, 42-44] Zanamivir **3** (Sigma-Aldrich, USA) and Cytochalasin D (Sigma-Aldrich, USA) were obtained from commercial sources and used as provided. Stock solutions for all the compounds, except cytochalasin D, were made using ultra-pure water (18.2 MΩ). Stock solutions of cytoD were made in DMSO, and the final concentration of DMSO in all samples was less than 0.1%. The FITC-conjugated *Maackia amurensis* lectin (Bioworld, USA), FITC-conjugated *Sambucas nigra* agglutinin (EY Labs, USA), and AF647-conjugated PNA (Thermo Fischer, USA) were obtained from commercial sources.

3.5.3 Neuraminidase enzymes

Recombinant NEU3 and NEU4 were prepared and purified from *E. coli* following an established procedure.[81] *Clostridium perfringens* neuraminidase was purchased (Sigma-Aldrich) and was found to consist predominantly of the NanI isoenzyme based on molecular weight determined from SDS-PAGE.[36] NEU

activity was determined using a standard 4MU-NANA assay, where 1 relative unit (RU) was defined as 1 μmol 4MU-NANA substrate cleavage per min.

3.5.4 Migration Studies

Cell migration studies were performed using an Oris 96 well plate assay kit (Platypus Technologies, USA). Each well of the plate was coated using 100 μL of fibronectin (10 μg/mL in PBS, Calbiochem, USA) for 2 h and 100 μL of IgG-free BSA (200 μg/mL in PBS) for 1 h. After removal of BSA, stoppers were placed in each well. Cells were placed into each well (100 μL of 50 x 10^4 cells/mL) and incubated for 18 h to achieve 80%-90% confluence. The stoppers were removed, cells were gently washed with PBS, and medium supplemented with treatment conditions or medium alone was reintroduced in each well. Wells were imaged under bright field illumination using a 5x objective with a Nikon T1 Eclipse inverted microscope. The plate was incubated for 21 h at 37 °C in a humidified incubator, and wells were imaged again and analyzed with Image J software. Each condition was repeated on different days to account for intra- and inter-day variability, and buffer and cytoD controls were used on every plate. Migration rates were normalized to the intra-day buffer control. Normalized replicates were then pooled together for statistical analysis. Migration data were normalized using the following equation: Normalized migration, $r = \left(\frac{\mathrm{A0} - \mathrm{A21}}{M_B}\right) \times 100$. Where A_0 was the cell-free zone at zero hours (μm^2), A_{21} was the area covered during 21 h incubation (μm^2), and M_B was the mean area covered by cells treated with only medium (μm^2).

3.5.5 Western blotting

Cells lysates were prepared in TBST buffer (1% Tween 20, 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM $MgCl_2$, and protease and phosphatase inhibitors), clarified by centrifugation, and boiled in Laemmli sample buffer. Lysates were separated by 10% SDS–PAGE. Lysate protein concentration was determined by bicinchoninic acid assay (BCA assay kit, Thermofisher, USA). Gels were transferred to nitrocellulose, blocked for 1 h at room temperature in TBS containing 5% milk (Biorad, USA), and then incubated in 1% TBST overnight at 4 °C with antibodies against beta-actin (anti-β actin antibody; 1:5,000; Abcam, ab8227), β1 integrin (Anti-integrin beta-1 antibody; 1: 5,000; clone EP1041Y, Abcam), α5 integrin (Anti-Integrin alpha-5 antibody; 1:1,000; clone EPR7854, Abcam), NEU1 (mouse monoclonal to human anti-neuraminidase 1; 1:1,000; Santa Cruz, clone F-8), and NEU3 antibody (mouse monoclonal to human anti-neuraminidase 3, clone 11b; 1:3,000; MBL), followed by the appropriate HRP conjugated secondary goat anti-rabbit antibody (1:5,000; Abcam) or goat anti-mouse antibody (1:3000; Biorad). Western blots were developed by ECL (BioRad, USA) according to the manufacturer's instructions.

3.5.6 Analysis of gangliosides in PC-3 cells treated with inhibitors using LC-MS

Expression and purification of EGCase was performed based on the reports of Albrecht et al.,[56] using a gene encoding recombinant EGCase I with a His-tag in a pET30 vector. The protein was purified on a Ni-NTA superflow column, concentrated, and stored at 4 °C. The activity of EGCase I was tested using ganglioside substrate GM3. One unit of EGCase I was defined as the amount of

enzyme that hydrolyzes 1 μmol of GM3 per minute at 30 °C. Ganglioside extraction and purification was performed based on previous reports.[82] Briefly, 1 x 10^6 cells were centrifuged to a pellet and homogenized in ice cold water (4 mL/g). Methanol and chloroform were added after vigorous mixing to a final ratio of 4:8:3 chloroform:methanol:water (v/v/v). This mixture was centrifuged (1500 RPM for 15 mins), and the supernatant was recovered and diluted with 0.173 volumes of water. After mixing, the suspension was centrifuged again, the upper phase was recovered, and transferred to a fresh tube. The sample was then purified on a SepPak C18 cartridge and evaporated to dryness under a stream of nitrogen. The final extract was re-suspended in sodium acetate buffer (50 mM, pH 5.2) containing 1 mg mL^{-1} sodium cholate. Gangliosides were then incubated for 18 hours at 30 °C with 0.086U EGCase to release the corresponding glycans. EGCase released glycans were labeled with anthranilic acid (30 mg anthranilic acid, 20 mg boric acid, 40 mg sodium acetate, and 45 mg sodium cyanoborohydride) at 80 °C for 45 mins, with an internal standard (maltose) added. The sample was purified on a discovery DPA-6S amide-HILIC column as described,[55] and reduced under vacuum. Labelled glycans were analyzed by LC-MS using an Agilent 1200 SL HPLC system and a normal-phase column (Accucore-150-Amide-HILIC, 2.6 μm, 2.1 x 150 mm, Thermo Fisher). The fluorescence detector was set to monitor at excitation 320 nm, emission 420 nm and all chromatography was performed at 40 °C. The binary solvent system followed a linear gradient with a flow rate of 0.4 mL min-1 (Solvent A: 100 mM ammonium formate, pH 4.45; Solvent B: acetonitrile). Quantification of glycolipid concentrations was calculated by reference to an

internal standard. Data analysis was performed using the Agilent MassHunter Qualitative Analysis software package version B.07.01.

3.5.7 Endocytosis of β1 integrin

Biotin-based assays were performed as previously described[59] with slight modifications. MDA-MB-231 or PC-3 cells were grown in 10% FBS-containing medium on 6 cm dishes to 80% confluence. The cells were placed on ice and washed once with cold PBS. Cell surface proteins were labeled with 0.8 mg mL^{-1} of EZ-link cleavable sulfo-NHS-SS-biotin (Thermo Scientific, USA) in for 60 min at 4 °C. Unbound biotin was washed away with cold medium, and pre-warmed 10% serum-containing medium with or without treatments were added to cells. The biotin-labelled surface proteins were allowed to internalize at 37 °C for 30 min, after which the cells were placed quickly back on the ice with cold medium. The remaining biotin at the cell surface after internalization was removed with GSH buffer (75 mM sodium chloride, 1 mM magnesium chloride, and 0.1 mM calcium chloride, 50 mM GSH, 80 mM sodium hydroxide, and 10% FBS) for 30 min at 4 °C, followed by repetitive washes with cold PBS. The cells were lysed by scraping in lysis buffer (150 mM sodium chloride 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0, phosphatase and protease inhibitor cocktail) and incubation at 4 °C for 30 min. The lysate was clarified by ultra-centrifugation at 18,000 x g for 10 min. Supernatants were collected and biotinylated integrin was measured by western blot.

3.5.8 Cell viability

Toxicity of compounds was assayed under similar conditions that were used in migration studies. Briefly, wells in a 96 well plate were charged with 100 µL of 50 x 10^4 cells mL^{-1} cells and incubated for 18 h in 5% CO_2 at 37 °C in a humidified incubator. Cells were then treated with the indicated conditions for 21 h. The final concentration of the compounds was the same as that used for migration studies. After incubation, 20 µL of MTS solution (Promega, USA) was added to each well and incubated for 2 h. The absorbance of soluble formazan produced by viable cells from MTS was measured at 490 nm using SpectraMax M2 (Molecular Devices, USA) plate reader. For each condition, the experiment was conducted with replicates on different days to consider the intra- and inter-day variabilities. Absorbance for each replicate of conditions was normalized to that of intra-day buffer control.

3.5.9 Measurement of cell surface sialic acid

Changes in cellular SGC on MDA-MB-231 and PC-3 cell lines after treatment with neuraminidase inhibitors and bacterial neuraminidase enzymes was assessed using flow cytometry. Cells were treated with 10 mM EDTA in PBS for 1.5 h to detach cells from culture plates and incubated for 18 h in fresh growth media. Cells were then treated with hNEU inhibitors in growth medium and incubated for 21 h. For NanI-treated cells, after harvesting cells, 0.06 mg mL^{-1} NanI was used to treat cells for 2 h. Cells (1 mL, 10 x 10^4 cells mL^{-1}) were labeled by incubation with AF 647-PNA (20 µg mL^{-1}), FITC-SNA (20 µg mL^{-1}), and FITC-MAL (5 µg mL^{-1}) for 30 mins separately. Cells were analyzed using a BD Accuri C6 flow cytometer.

Doublet discrimination and gating was used to select monodisperse cells. Experiments were conducted with 4-6 replicates on different days to consider the intra- and inter-day variabilities. Fluorescence of each replicate conditions was normalized to that of the intra-day buffer control.

3.6 **References**

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4. Regulation of inflammation in vivo by NEU1 [1]

[1] Compounds used in these studies were synthesized and characterized by Tianlin Guo; In vivo experiment were carried out in the lab of Prof. Alexey V. Pshezhetsky (University de Montreal); Ekatrina Demina contributed in the in vivo studies; Suzzane Summarani and Prof. Ali Ahmed (University de Montreal) provided FACS analysis of leukocyte subsets. IHC was performed by Sullen Lamb, Histology Lab Services, U of Alberta. Some authors (MAH, CWC, AVP) are inventors on a provisional patent filed related to this work.

4.1 **Introduction**

The inflammatory cascade is an important component of acute inflammation, as it provides an essential mechanism for trafficking of leukocytes to the site of inflammation.[1] This process provides a rapid response to infection by regulating the permeability of the vascular compartment, activation of endothelial cells, binding of leukocytes to the endothelium, extravasation of leukocytes to the site of inflammation, as well as activation of macrophages, platelets, and other clotting factors.[2] Acute inflammation can be triggered by recognition of pathogen associated molecular patterns (PAMPs), such as the lipopolysaccharide (LPS) of gram negative bacteria.[3] The presence of LPS, or other PAMPs, is recognized by receptors such as the toll-like receptor 4 (TLR-4) leading to initiation of the inflammatory cascade and leukocyte recruitment.[4] The glycosylation state of cellular receptors, including TLR-4, is known to be regulated by endogenous neuraminidase enzymes (NEU, or sialidases).[5, 6] Notably, there are four isoenzymes of NEU with distinct substrate specificity, tissue expression, sub-cellular localization, and roles in pathogenesis.[7] The role of these individual NEU isoenzymes in leukocyte recruitment has rarely been investigated directly *in vivo*.

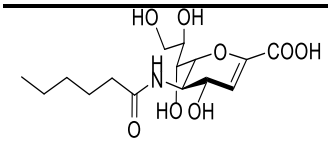
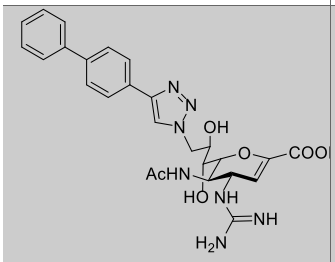
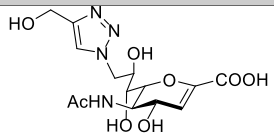
Neuraminic acid (Neu5Ac, or sialic acid) residues are known to be important in multiple steps of the inflammatory cascade. Selectins, which mediate leukocyte capture and rolling, require a minimal tetrasaccharide ligand, sialyl-Lewis^X (sLe^X, CD15s), for binding.[8] Endogenous NEU are known to modify this epitope, thus disrupting selectin-ligand interactions.[9] The firm adhesion step of the cascade is mediated by leukocyte β2 integrins LFA-1 (CD11a/CD18) and MAC-1

(CD11b/CD18). Activation epitopes of LFA-1 have been found to be modulated by endogenous NEU activity.[10] NEU enzymes can increase adhesion of polymorphonuclear leukocyte (PMN) adherence to endothelium and also increase migration through the endothelium and the site of inflammation.[11, 12] Activated PBMCs have been shown to increase production of inflammatory cytokines such as IFN-γ through action of NEU1 or NEU3.[13] The effect of cytokine production (e.g. IL-6, IL-12p40, and TNF-α) in dendritic cells has also been reported to be regulated by NEU1 and NEU3 activity.[14] Together, these findings suggest that endogenous NEU activity is involved at multiple points along the inflammatory cascade. Neuraminidase enzymes have been investigated in different inflammatory models such as pulmonary fibrosis,[15] LPS-induced acute lung injury,[16] and sepsis.[17,18,19]

In this study we systematically investigated the role of individual mammalian NEU isoenzymes in acute inflammation using a murine model. We adopted a six-day air pouch model of acute inflammation[20] using NEU KO mice challenged with an LPS stimulus. We hypothesized that individual NEU isoenzymes would have different effects on inflammation, specifically on the recruitment of leukocytes to the site of inflammation. We observed that the inflammatory response to LPS had distinct modulation from NEU isoenzymes, with NEU1 and NEU3 isoenzymes acting as positive regulators and NEU4 acting as a negative regulator. These results demonstrate that NEU isoenzymes have important and distinct roles in the regulation of immune cells, and indicate that further study is required to completely

understand the mechanisms of each enzyme and their involvement in normal and pathogenic inflammation.

Table 4.1: hNEU inhibitors used in this study.

Structure	Identifiers	IC_{50} [μM][d]				Selectivity[e]
		NEU 1	NEU 2	NEU 3	NEU 4	
	CG146001 [a,21]	0.42	15	210	440	NEU1 (36X)
	CG226002 [b,22]	>500	6	0.58	6	NEU3 (10X)
	C9-4HMT-DANA[c, 23] 3	>500	>500	80	0.16	NEU4 (500X)

a, Referred to as compound 11d in original citation.[21]

b, Referred to as compound 8b in original citation.[22]

c, Referred to as compound 6 in original citation.[23] Referred to here as C9-(4-hydroxymethyltriazolyl)-DANA (C9-4HMT-DANA).

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.

4.2 Results

4.2.1 Inflammatory response to LPS reveals involvement of neuraminidase enzymes in the recruitment of leukocytes

We used a murine air pouch model to investigate the effect of neuraminidase enzymes on inflammation.[20] An air pouch was created by subcutaneous (*sc*) injection of sterile air on the backs of WT (C57BL6) and NEU1 KO, NEU3 KO, NEU4 KO, and NEU3/4 DKO animals (see Materials & Methods). After a short period (9 h), the pouch was injected with either saline or saline containing LPS to simulate a bacterial infection. Subsequent to an incubation period, the air pouch was washed with sterile saline to harvest cells, which were then counted by flow cytometry (**Figure 4.1, Table 4.2**). Saline treatment provided a comparison of basal inflammation between WT and genotypic mice and differences were unremarkable (**Figure 4.1B**). In contrast, LPS treatment showed significant differences that were dependent upon NEU expression (**Figure 4.1C**). In the case of NEU1 KO animals, leukocyte counts were reduced by 4-fold ($79 \pm 9 \times 10^4$ cells/pouch) as compared to the WT LPS response ($326 \pm 29 \times 10^4$ cells/pouch). The NEU3 KO animals showed a reduced response to LPS as compared to the saline control; however, this was not significantly different from WT response to LPS. The NEU4 KO ($1371 \pm 230 \times 10^4$ cells/pouch) and NEU3/4 DKO ($992 \pm 140 \times 10^4$ cells/pouch) animals demonstrated a significant increase of between 3-4 fold as compared with the WT LPS response. These results suggested that NEU4 acted as a negative regulator of leukocyte recruitment, while NEU1 acted as a positive regulator.

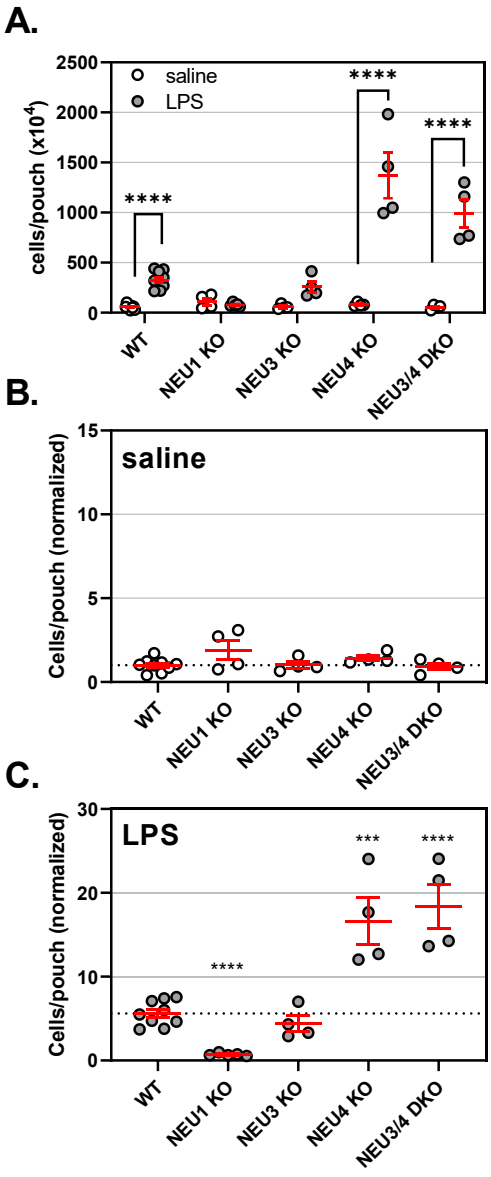


Figure 4.1. Leukocyte counts from the air pouch model. An air pouch was formed after injection of sterile air for each genotype. The pouch was then injected with saline or LPS and incubated for 9 h. Mice were then sacrificed, the pouch exudate was collected, and cells were counted by FACS. A. Cell counts are presented for saline (○) or LPS (●) treatment. B. Cell counts for saline treatment are plotted normalized to WT controls (58 x 10^4 cells/pouch). C. Cell counts for LPS treatment are plotted normalized to WT controls as in B. The dashed line indicates the average LPS response of the WT animal. Data are presented as mean ± SEM and comparisons were made to the WT controls using the Students t-test (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.005$; ****, $p \leq 0.0001$).

Table 4.2. Leukocyte raw cell counts observed in the air pouch model.

	Saline (cells x10^4)			LPS (cells x10^4)		
	Mean ± SEM	*p*	N	Mean ± SEM	*p*	N
WT C57BL6	58 ± 8		9	326 ± 29		9
NEU3 4 DKO	54 ± 12		4	992 ± 141	****	4
NEU 4 KO	83 ± 9		4	1371 ± 229	****	4
NEU 3 KO	59 ± 12		4	259 ± 54		4
NEU1 KO	111 ± 34		4	79 ± 9	****	5
Inh 1	72 ± 16		4	357 ± 111		4
Inh 2	100 ± 26		4	189 ± 40	**	4
Inh 3	172 ± 43	**	4	433 ± 49		3

Values were compared to control using a Student's t-test (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.005$; ****, $p \leq 0.0001$).

Table 4.3: Summary of changes to plasma cytokines in NEU1 and NEU4 KO animals.

Model	Activated[a]	Basal activated[b]	Attenuated[c]
NEU1		IL-1α, IL-15	G-CSF, IL-6, INFγ, CCL5
NEU4	IL-21		G-CSF, CCL5

a. Activated cytokine levels were defined as having a *significant difference between saline and LPS treatment in the control*, and showing a significant *increase between control and model LPS stimulation*.

b. Basal activation levels were defined as having *no significant difference between saline and LPS treatment in control*, and a *significant increase in saline* for the model relative to saline control.

c. Attenuated cytokine levels were defined as having a *significant difference between saline and LPS treatment in the control*, and showing a *significant decrease between control and model LPS stimulation*.

Based on these findings we sought to investigate the effect of specific inhibitors of neuraminidase isoenzymes in the same inflammation model (**Table 4.1; Figure 4.2**). Inhibitors were dosed at 1 mg/kg body weight for each animal. We observed that treatment of mice with a selective NEU1 inhibitor 1 had no significant effect on leukocyte counts after LPS treatment (357 ± 111 x 10^4 cells/pouch) as compared to saline-treated mice. Treatment with a selective NEU3 inhibitor 2 significantly reduced the LPS response of the WT mouse (189 ± 40 x 10^4 cells/pouch) relative to saline controls. Animals treated with a selective NEU4 inhibitor 3 did not affect leukocyte response to LPS (433 ± 49 x 10^4 cells/pouch); however, this treatment did result in a significant increase in leukocyte counts for saline-treated mice (172 ± 43 x 10^4 cells/pouch). Our inhibitor results were not conclusive for NEU1, but are consistent with NEU3 as a positive regulator and NEU4 as a negative regulator of leukocyte recruitment. The inhibitors showed differential effects on leukocyte subsets (*vide infra*), which may explain the divergence of these results from the genotypic animals. This may be the result of the compound pharmacokinetics or membrane permeability. Therefore, we would caution that these results require further study and may be dependent on the dosing regimen. Future experiments will be needed to confirm these results.

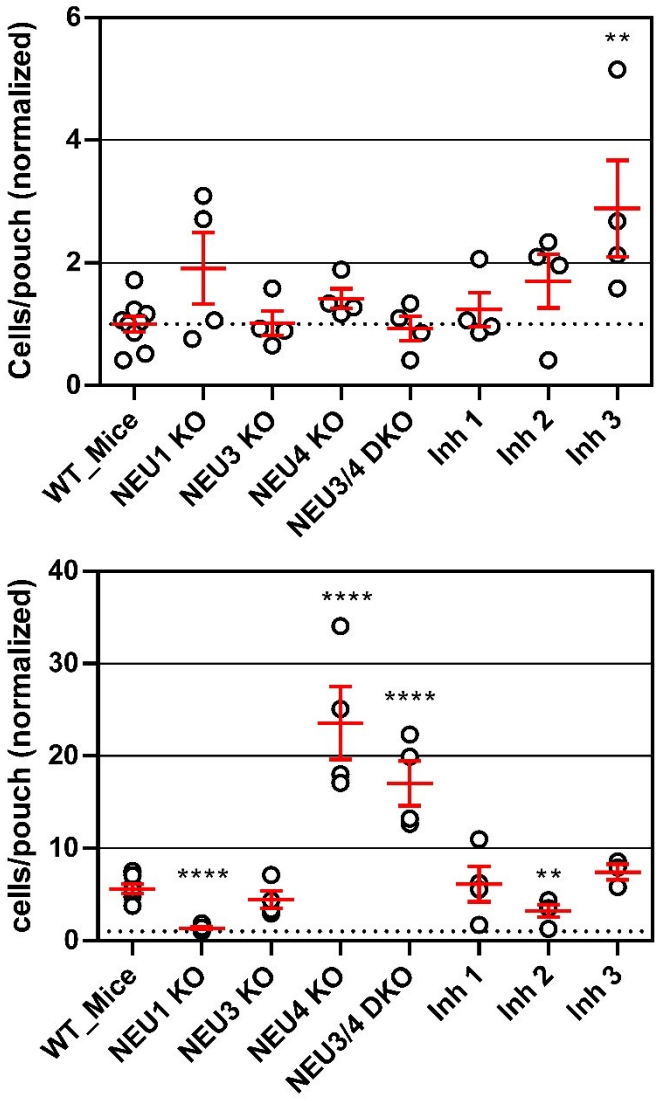


Figure 4.2: Leukocyte counts from the air pouch model. An air pouch was formed after injection of sterile air for each genotype. The pouch was then injected with saline or LPS and incubated for 9 h. Mice were then sacrificed, the pouch exudate was collected, and cells were counted by FACS. A. Cell counts are presented for saline (○) or LPS (●) treatment. B. Cell counts for saline treatment are plotted normalized to WT controls (58 x 10^4 cells/pouch). C. Cell counts for LPS treatment are plotted normalized to WT controls as in B. The dashed line indicates the average LPS response of the WT animal. Data are presented as mean ± SEM and comparisons were made to the WT controls using the Students t-test (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.005$; ****, $p \leq 0.0001$).

4.2.2 Leukocyte subset response to LPS in neuraminidase deficient animal models

Based on our measurements of total leukocyte counts in the air pouch model of NEU-deficient animals, we sought to determine the leukocyte subsets influenced by these enzymes. To determine the leukocyte subsets, we collected and stained the pouch exudate with specific antibodies and quantified them using FACS. Normalized cell counts for selected leukocyte subsets are shown in **Figure 4.3** (also see **Table 4.4**). We observed that the major populations found in the air pouch after LPS treatment were primarily monocytes (MO), neutrophils (NE), natural killer (NK), and macrophage (Mφ) cells. T and B cells counts were low in both conditions, and no remarkable changes were noted for any of the NEU-deficient animals.

Table 4.4. Leukocyte subsets in the air pouch model by FACS.

MONOCYTES	Saline (cells x10^4)		LPS (cells x10^4)	
	Mean ± SEM	N	Mean ± SEM	N
WT C57BL6	7 ± 2	8	110 ± 24	8
NEU3 4 DKO	20 ± 6	5	490 ± 94	5
NEU 4 KO	36 ± 5	4	771 ± 150	4
NEU 3 KO	34 ± 7	4	91 ± 12	3
NEU1 KO	59 ± 12	4	35 ± 7	5

NEUTROPHILS	Saline (cells x10^4)		LPS (cells x10^4)	
	Mean ± SEM	N	Mean ± SEM	N
WT C57BL6	5 ± 1	8	57 ± 9	8
NEU3 4 DKO	7 ± 2	5	179 ± 47	5
NEU 4 KO	10 ± 1	4	507 ± 111	4
NEU 3 KO	13 ± 5	4	20 ± 2	3
NEU1 KO	28 ± 11	4	10 ± 2	5

MACROPHAGES	Saline (cells x10^4)		LPS (cells x10^4)	
	Mean ± SEM	N	Mean ± SEM	N
WT C57BL6	2 ± 1	8	11 ± 3	8
NEU3 4 DKO	3 ± 1	5	20 ± 8	5
NEU 4 KO	3 ± 1	4	13 ± 3	4
NEU 3 KO	4 ± 1	4	8 ± 2	3
NEU1 KO	2 ± 1	4	1 ± 1	5

NK CELLS	Saline (cells x10^4)		LPS (cells x10^4)	
	Mean ± SEM	N	Mean ± SEM	N
WT C57BL6	9 ± 5	4	38 ± 9	4
NEU3 4 DKO	5 ± 2	5	70 ± 22	5
NEU 4 KO	3 ± 1	4	95 ± 6	4
NEU 3 KO	14 ± 6	4	18 ± 6	4
NEU1 KO	1 ± 1	2	2 ± 0.1	2

B Cells	Saline (cells x10^4)		LPS (cells x10^4)	
	Mean ± SEM	N	Mean ± SEM	N
WT C57BL6	2 ± 1	4	15 ± 7	4
NEU3 4 DKO	4 ± 1	5	94 ± 60	5
NEU 4 KO	4 ± 1	4	3 ± 1	4
NEU 3 KO	2 ± 1	4	6 ± 2	4
NEU1 KO	3 ± 3	2	1 ± 0.1	2

T CELLS	Saline (cells x10^4)		LPS (cells x10^4)	
	Mean ± SEM	N	Mean ± SEM	N
WT C57BL6	5 ± 4	4	30 ± 20	4
NEU3 4 DKO	5 ± 1	5	154 ± 46	5
NEU 4 KO	5 ± 1	4	34 ± 10	4
NEU 3 KO	12 ± 3	4	34 ± 8	4
NEU1 KO	2 ± 2	2	4 ± 1	2

Results expressed as mean ± standard error of the mean (SEM).

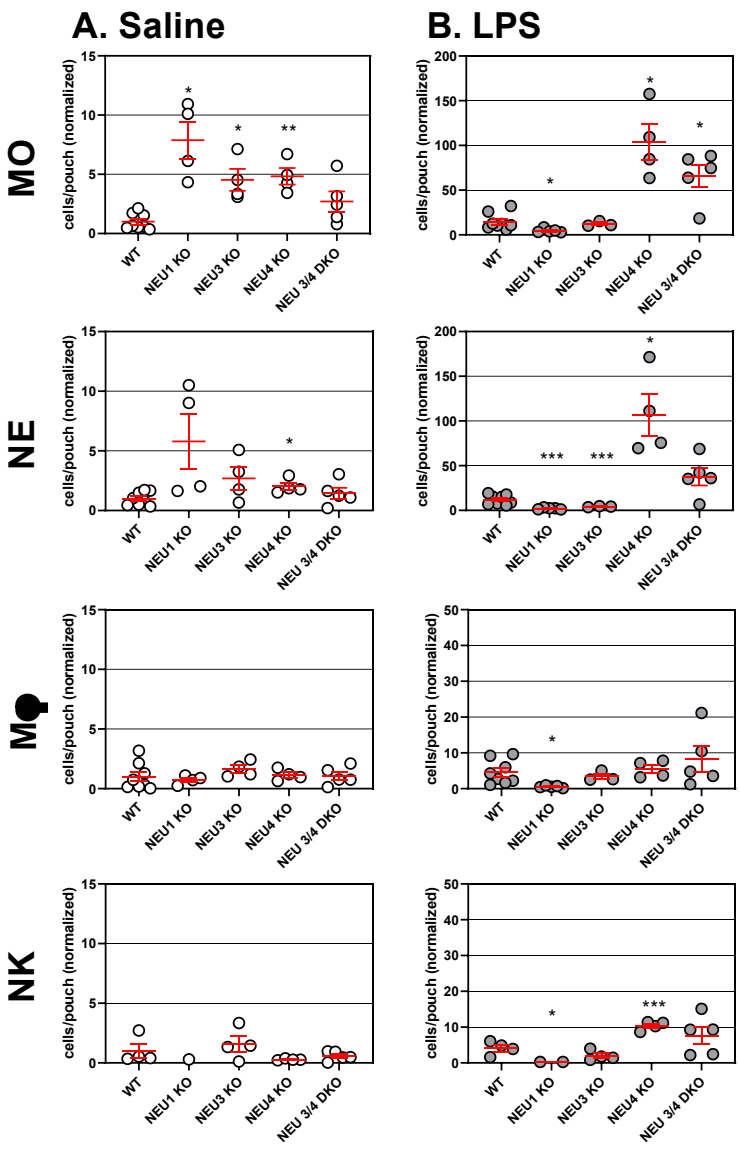


Figure 4.3. Leukocyte populations in the air pouch model. Leukocytes collected from animals after A. saline (○), or B. LPS (●) treatment were counted by FACS and identified based on antibody markers as monocyte (MO), neutrophil (NE), macrophage (Mφ), or natural killer (NK) cells. The air pouch exudate was collected after 9 h from WT, NEU1 KO, NEU3 KO, NEU4 KO and NEU34 DKO mice. The data is presented as cell counts normalized to the WT saline controls (monocyte, 7.4; neutrophil, 4.7; macrophage, 2.3; NK, 9.1 x10^4 cells/pouch) mean ± SEM, and conditions were compared to control using Students t-test (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.005$; ****, $p \leq 0.0001$). Raw cell counts are presented in Table 4.4.

Comparisons of the cell counts for each subset revealed cell-type specific influences of the NEU enzymes. We found that NEU1 KO animals had consistently reduced levels of monocyte, neutrophil, and NK cells (between 3-10 fold as compared to WT) after LPS treatment. These animals also displayed an increased monocyte count from saline treatment. In contrast, NEU4 KO animals had elevated counts of monocytes, neutrophils, and NK cells after LPS treatment; as well as elevated monocyte and neutrophil counts in saline controls (between 8-9 fold as compared to WT). The NEU3 KO animals had reduced counts of neutrophils after LPS treatment (3-fold as compared to WT) and elevated monocyte counts in saline controls. NEU3/4 DKO animals showed moderate elevation of monocyte counts with LPS treatment. These results are generally consistent with NEU1 acting as a positive regulator of monocyte, neutrophil, macrophage, and NK cell response to LPS. Moreover, NEU4 acted as a negative regulator of monocyte, neutrophil, and NK cell response to LPS. The results for NEU3 suggest that it may play a role as a positive regulator of neutrophils after LPS stimulation.

4.2.3 Effect of Neuraminidase enzyme inhibitors on leukocyte subsets

We also analyzed the response of leukocyte subsets in the presence of inhibitors directed at the human NEU enzymes (**Table 4.5**, **Figure 4.4**). We counted leukocytes harvested from the air pouch model as above after three injections of compounds **1**, **2**, or **3** at 1 mg/kg body weight of mice (delivered in three doses at 48, 24, and 9 h before sacrifice.) Compound **1**, a selective inhibitor of NEU1, was able to block the LPS response of WT neutrophils, macrophages, and NK cells; however, monocytes were not affected. This effect is consistent with

the reduced NEU1 KO response to LPS. Compound **2**, a selective inhibitor of NEU3, was able to block the LPS response of WT monocytes, neutrophils, macrophages, and NK cells. Finally, compound **3**, a selective inhibitor of NEU4, blocked the LPS response of macrophages and NK cells. Interestingly, saline and inhibitor **3** conditions showed elevated levels of monocytes and neutrophils. These findings further support that NEU1 and NEU3 likely act as positive regulators of leukocytes, while NEU4 acts as a positive regulator. Furthermore, these results indicate that inhibitors of these enzymes should be investigated as anti-inflammatory therapeutics. While these results are intriguing and add support to our findings in KO animals, further work is required to confirm optimal dosing of the inhibitors and to confirm their selectivity *in vivo*.

Table 4.5. Leukocyte populations in the air pouch model after inhibitor treatment by FACS.

MONOCYTES	Saline (cells x10^4)		LPS (cells x10^4)	
	Mean ± SEM	N	Mean ± SEM	N
WT C57BL6	7 ± 2	8	75 ± 8	8
Inh 1	17 ± 4	4	157 ± 48	5
Inh 2	37 ± 13	4	78 ± 22	4
Inh 3	25 ± 1	3	182 ± 46	3

NEUTROPHILS	Saline (cells x10^4)		LPS (cells x10^4)	
	Mean ± SEM	N	Mean ± SEM	N
WT C57BL6	5 ± 1	8	57 ± 9	8
Inh 1	7 ± 1	4	121 ± 56	4
Inh 2	26 ± 7	4	42 ± 9	4
Inh 3	22 ± 7	4	146 ± 41	3

MACROPHAGES	Saline (cells x10^4)		LPS (cells x10^4)	
	Mean ± SEM	N	Mean ± SEM	N
WT C57BL6	2 ± 1	8	11 ± 3	8
Inh 1	2 ± 1	5	2 ± 1	5
Inh 2	2 ± 1	4	1 ± .2	4
Inh 3	2 ± 1	4	5 ± 2	3

NK CELLS	Saline (cells x10^4)		LPS (cells x10^4)	
	Mean ± SEM	(N)	Mean ± SEM	(N)
WT C57BL6	4 ± 1	(3)	38 ± 9	(4)
Inh 1	13 ± 2	(4)	24 ± 8	(4)
Inh 2	3 ± 1	(4)	7 ± 1	(4)
Inh 3	30 ± 10	(4)	65 ± 31	(3)

B CELLS	Saline (cells x10^4)		LPS (cells x10^4)	
	Mean ± SEM	(N)	Mean ± SEM	(N)
WT C57BL6	2 ± 1	(4)	15 ± 7	(4)
Inh 1	5 ± 2	(2)	4 ± 2	(2)
Inh 2	1 ± 1	(4)	1 ± 1	(4)
Inh 3	2 ± 1	(4)	7 ± 3	(4)

T CELLS	Saline (cells x10^4)		LPS (cells x10^4)	
	Mean ± SEM	(N)	Mean ± SEM	(N)
WT C57BL6	5 ± 4	(4)	30 ± 20	(4)
Inh 1	31 ± 17	(4)	10 ± 4	(4)
Inh 2	83 ± 28	(4)	171 ± 42	(4)
Inh 3	48 ± 13	(4)	161 ± 85	(3)

Results expressed as mean ± standard error of the mean (SEM).

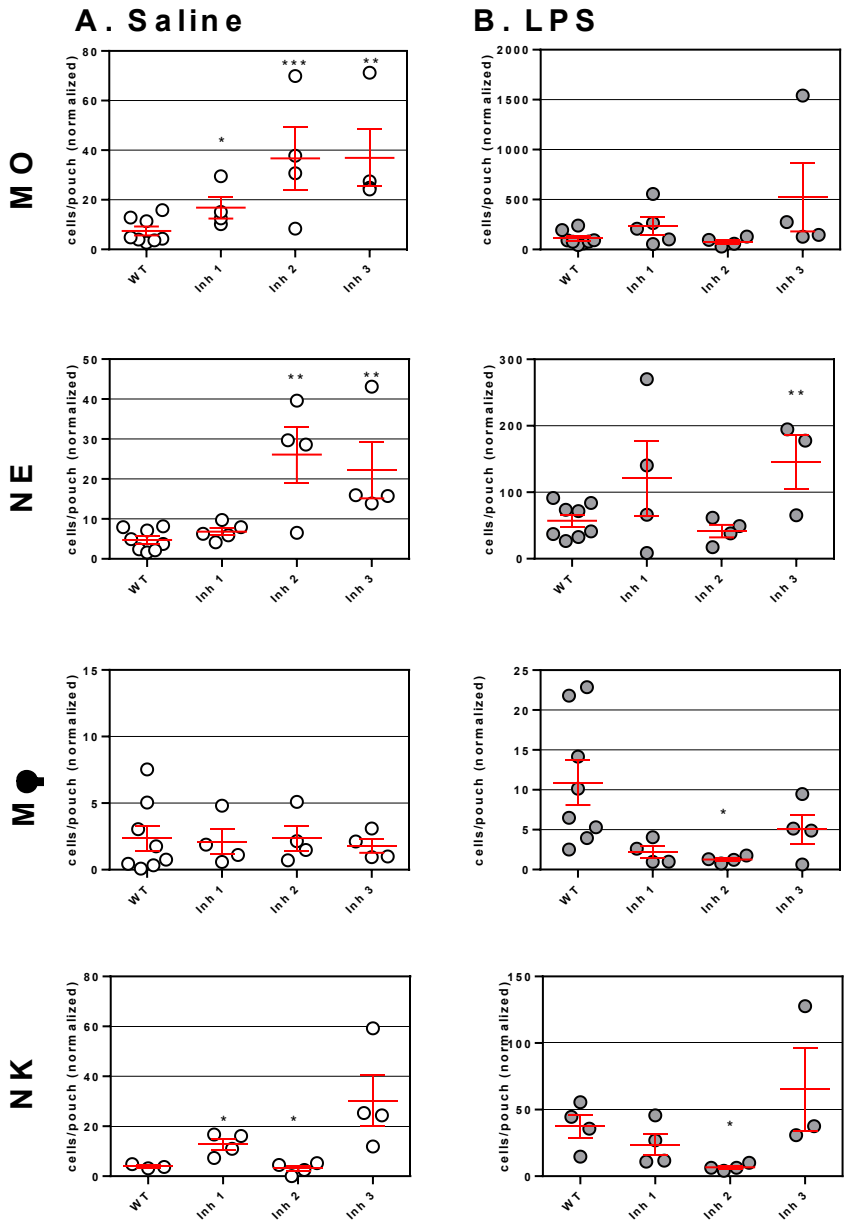


Figure 4.4. Leukocyte subsets in the air pouch model after treatment with neuraminidase inhibitors. Leukocytes collected from animals were counted by FACS and identified based on antibody markers. Cell types were A) Monocytes, B) Neutrophils, C) Macrophages, and D) NK cells. Animals were WT only and inhibitor treatments were at 1 mg/kg. The air pouch exudate was collected after 9 h. The data is presented as mean ± SEM, and conditions were compared to control using Students t-test (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.005$; ****, $p \leq 0.0001$).

4.2.4 Serum cytokine levels in NEU KO mice

Based on our findings above, we concluded that NEU-deficient animals have attenuated and activated effects on leukocyte recruitment. A potential explanation for these changes would be modulation of cytokine levels in the animals. We collected serum samples from the animals at the end point of the air pouch experiments and analyzed samples from NEU1 and NEU4 KO animals for a panel of 19 cytokines. Amongst them several of these cytokines including G-CSF, IL-1α, IL-1β, IL-15/IL-15R, IL-21, IL-6, INF-γ, IP-10, and RANTES (**Figure 4.5**, **Table 4.6**) showed significant changes in different genotypes. Other cytokines expression was unremarkable **(Figure 4.6)** and hence was not included in further discussion.

We evaluated the responses for each of these into three categories: showing no difference from control, *activated* (showing a significant different between saline and LPS treatment in control, along with a significant increase between control and LPS stimulation), *basal activated* (showing no significant difference between saline and LPS treatment in control, along with a significant increase in saline for the model relative to saline control), or *attenuated* (showing a significant difference between saline and LPS treatment in the control, along with a significant decrease between control and model LPS stimulation). The major observations from these data are summarized in **Table 4.3**. In NEU1 KO animals, we observed that IL-1α and IL-15 were basal activated; and G-CSF, IL-6, INF-γ, and CCL5 were all attenuated. We found that the NEU4 KO animals had activated levels of IL-21. The NEU4 KO animals also showed attenuated levels of G-CSF and CCL5.

While further experiments will be required to test if cytokine levels are a direct or indirect result of NEU-deficiencies, we note that there are clear differences

between cytokine levels of the two isoenzymes investigated here. We speculate that decreased leukocyte infiltration of the NEU1 KO animal is due to attenuated IL-6 levels, and increased leukocyte infiltration of the NEU4 KO is likely due to activated IL-21 levels.

IL-6 is a pleiotropic cytokine often shown to play pivotal roles in the transition to acquired immunity from innate immunity.[24] IL-6 trans-signaling is responsible for upregulation of cell adhesion molecules such as L-selectin (CD62L) in leukocyte[25] and ICAM-1, VCAM-1, and CD62E (E-Selectin) on endothelial cells, thereby enhancing the leukocyte transmigration.[26, 27] IL-6 has also shown to be involved in T cells and B cell recruitment and differentiation. Hence, the low IL-6 expression in NEU1 KO animals may be responsible for the observed attenuation of leukocyte infiltration.

IL-21 is a pro-inflammatory cytokine which has shown to enhance the inflammatory response and infiltration of leukocytes in the air pouch model after LPS stimulation.[28] The NEU4KO mice had increased IL-21 expression after LPS stimulation, which might explain the increased leukocyte infiltration observed in the experiments.

Table 4.6. Serum cytokine levels in mice.

		Saline (pg/mL)	N	LPS (pg/mL)	N
G-CSF/CSF-3	WT	5 ± 1	6	672 ± 97	6
	NEU1 KO	28 ± 2	6	163 ± 48	8
	NEU4 KO	22 ± 7	8	330 ± 39	8
IL-1 alpha	WT	15 ± 3	5	41 ± 4	6
	NEU1 KO	49 ± 7	6	27 ± 7	8
	NEU4 KO	21 ± 7	4	50 ± 12	5
IL-1 beta	WT	1 ± 0.1	5	7 ± 1	6
	NEU1 KO	7 ± 1	6	5 ± 1	8
	NEU4 KO	3 ± 1	4	7 ± 2	5
IL-15/IL-15R	WT	3 ± 1	6	23 ± 3	6
	NEU1 KO	21 ± 2	6	16 ± 4	8
	NEU4 KO	8 ± 2	7	23 ± 6	8
Il-21	WT	14 ± 5	3	24 ± 5	5
	NEU1 KO	29 ± 5	6	24 ± 5	6
	NEU4 KO	13 ± 4	3	63 ± 8	4
IL-6	WT	24 ± 8	7	557 ± 92	6
	NEU1 KO	63 ± 5	6	168 ± 59	8
	NEU4 KO	61 ± 9	8	599 ± 128	8
IFN-γ	WT	2 ± 1	3	5 ± 1	6
	NEU1 KO	4 ± 1	6	2 ± 1	8
	NEU4 KO	1 ± 0.5	8	4 ± 1	8
IP-10	WT	57 ± 3	6	732 ± 111	6
	NEU1 KO	35 ± 8	6	529 ± 62	8
	NEU4 KO	107 ± 10	8	620 ± 55	8
RANTES	WT	16 ± 2	6	924 ± 144	6
	NEU1 KO	54 ± 9	6	486 ± 90	6
	NEU4 KO	13 ± 4	6	362 ± 21	8
GM-CSF	WT	9 ± 2	5	35 ± 5	6
	NEU1 KO	34 ± 4	6	25 ± 6	8
	NEU4 KO	19 ± 5	6	31 ± 9	8
IL-10	WT	23 ± 5	6	43 ± 4	6
	NEU1 KO	33 ± 7	6	25 ± 7	8
	NEU4 KO	10 ± 4	5	25 ± 6	8
IL-12p40	WT	37 ± 7	6	49 ± 6	6
	NEU1 KO	42 ± 6	6	138 ± 61	8
	NEU4 KO	24 ± 3	8	46 ± 2	8
IL-25/IL-17	WT	74 ± 45	3	173 ± 29	6
	NEU1 KO	186 ± 18	6	114 ± 27	6
	NEU4 KO	84 ± 30	5	196 ± 55	6

		Saline (pg/mL)	N	LPS (pg/mL)	N
MCP-1	WT	10 ± 4	8	17 ± 2	6
	NEU1 KO	14 ± 2	6	12 ± 2	8
	NEU4 KO	6 ± 1	6	14 ± 2	8
MIP-2	WT	74 ± 45	3	173 ± 29	6
	NEU1 KO	186 ± 18	6	114 ± 27	6
	NEU4 KO	84 ± 30	5	196 ± 55	6
MIP-1 α	WT	74 ± 45	3	173 ± 29	6
	NEU1 KO	186 ± 18	6	114 ± 27	6
	NEU4 KO	84 ± 30	5	196 ± 55	6
MIP-1β	WT	74 ± 45	3	173 ± 29	6
	NEU1 KO	186 ± 18	6	114 ± 27	6
	NEU4 KO	84 ± 30	5	196 ± 55	6

Results expressed as mean ± standard error of the mean (SEM).

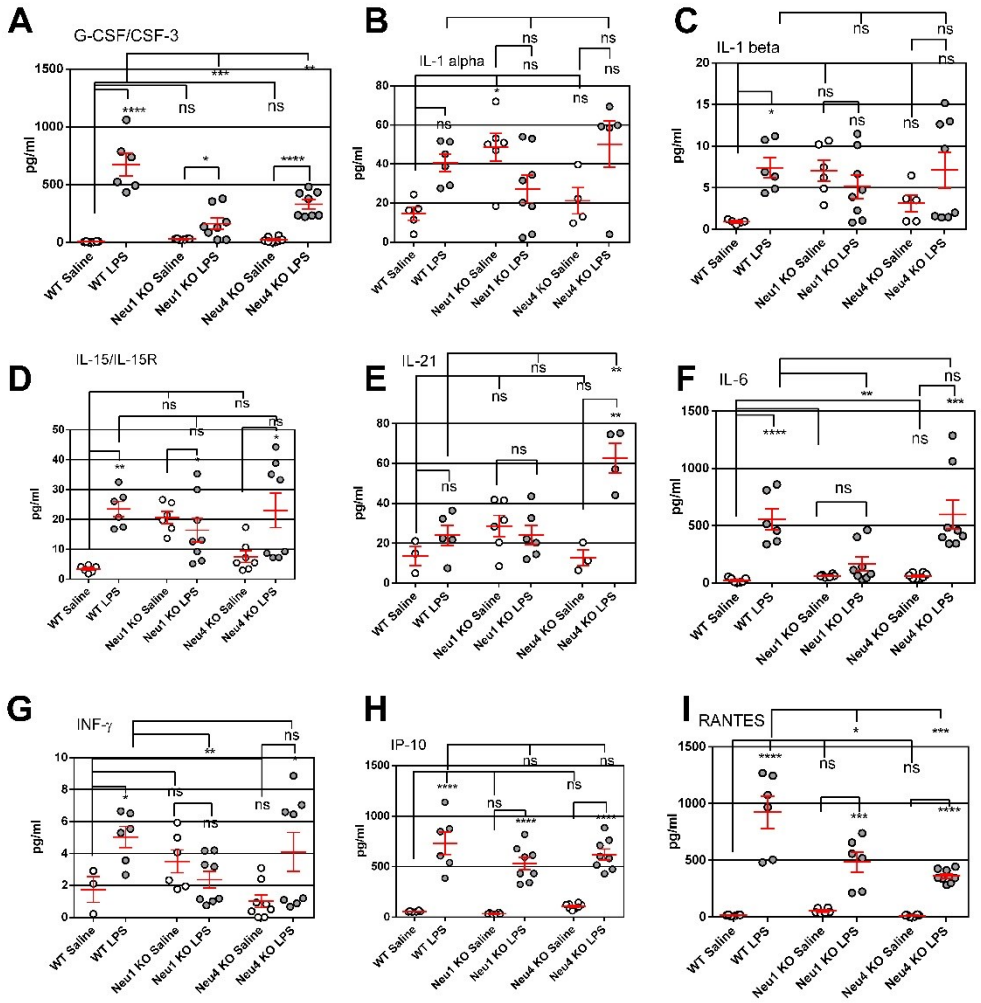


Figure 4.5. Serum cytokine levels in mice treated with LPS. Serum was collected from the sacrificed mice treated with saline or LPS in the air pouch model. The amount of systemic cytokines were then measured using Procata Plex custom immuno assay cytokine kit. The data are presented as mean ± SEM, and conditions were compared to control using a Students t-test (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.005$; ****, $p \leq 0.0001$).

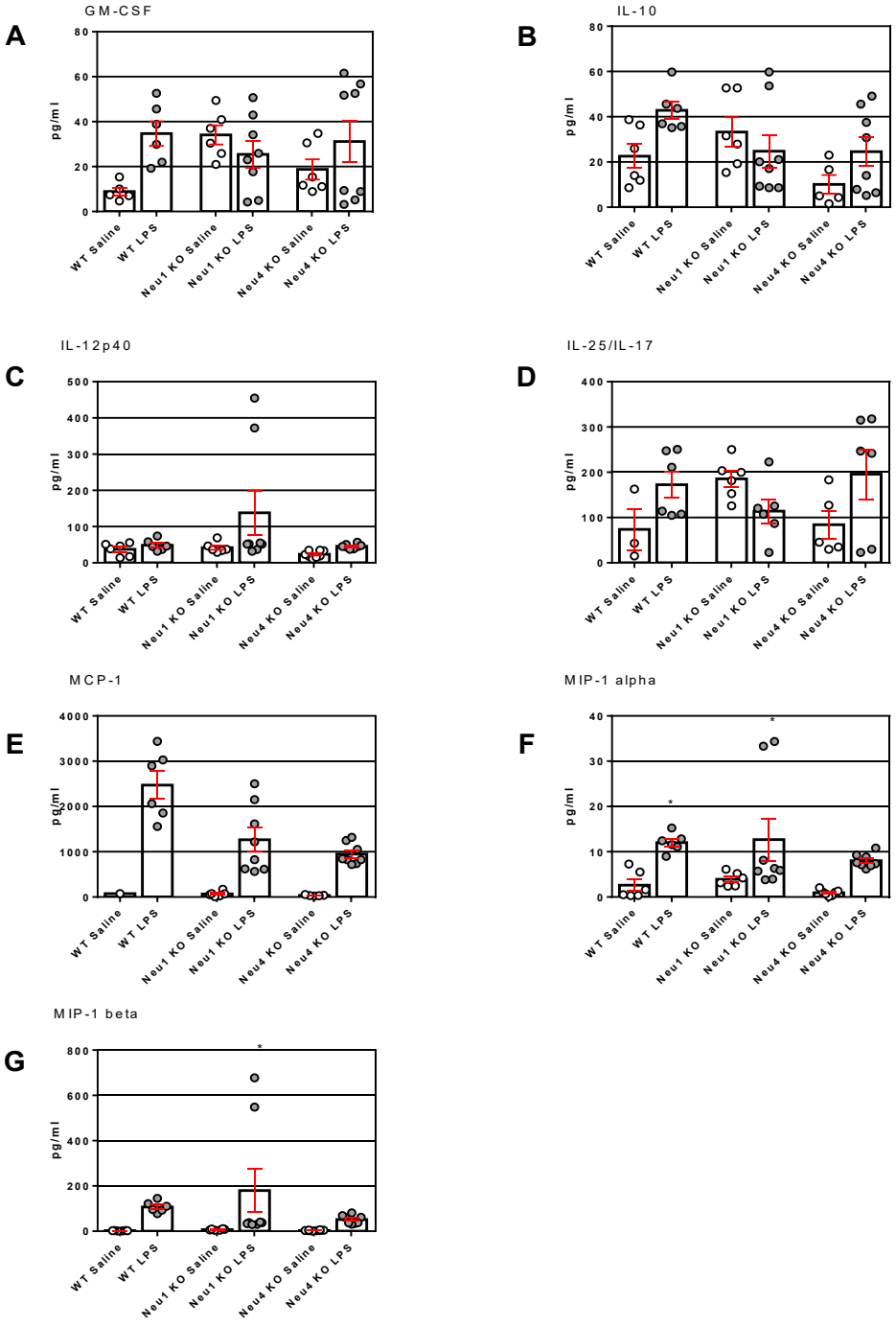


Figure 4.6. Serum cytokine levels in mice treated with LPS (Continued). Serum was collected from the sacrificed mice treated with saline or LPS in the air pouch model. The amount of systemic cytokines were then measured using Procata Plex custom immuno assay cytokine kit. The data are presented as mean ± SEM, and conditions were compared to control using a Students t-test (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.005$; ****, $p \leq 0.0001$).

4.2.5 Immunohistochemistry confirms the effects of NEU on leukocyte infiltration

To add further support to our conclusions regarding leukocyte infiltration, we obtained tissue samples from the air pouch models and analyzed regions of the dermis and muscle tissue for leukocyte infiltration (**Figure 4.7**, **Table 4.7, and Figure 4.8**). Sections of tissue were stained and we determined relative numbers of leukocytes for each condition shown. In the dermis layer we observed that the NEU1 KO animals had increased numbers of leukocytes after saline treatment relative to WT animals; however, this was not the case in muscle tissue. In both regions, NEU1 KO animals had reduced leukocyte counts after LPS treatment. Tissue from NEU3 KO animals showed an increase in leukocytes in the dermis of saline treated animals, and a reduced response to LPS in the dermis and muscle as compared to WT animals. NEU4 KO animals showed increased leukocyte counts in both dermis and muscle layers in both saline and LPS treatment relative to WT controls. This effect was attenuated in the NEU3/4 DKO animals: while saline treatment still showed elevated counts in dermis and muscle, LPS treatment showed either no difference from WT control (dermis) or a reduced count (muscle). This result suggests that NEU3 and NEU4 effects compete with each other. Together with our FACS data (**Figure 4.1**), these results confirm that NEU1 is a positive regulator of leukocyte LPS response, and NEU4 is a negative regulator. Leukocyte counts from tissues suggest that NEU3 can act as a positive regulator of LPS response, but this effect was not observed in the FACS analysis of pouch exudate.

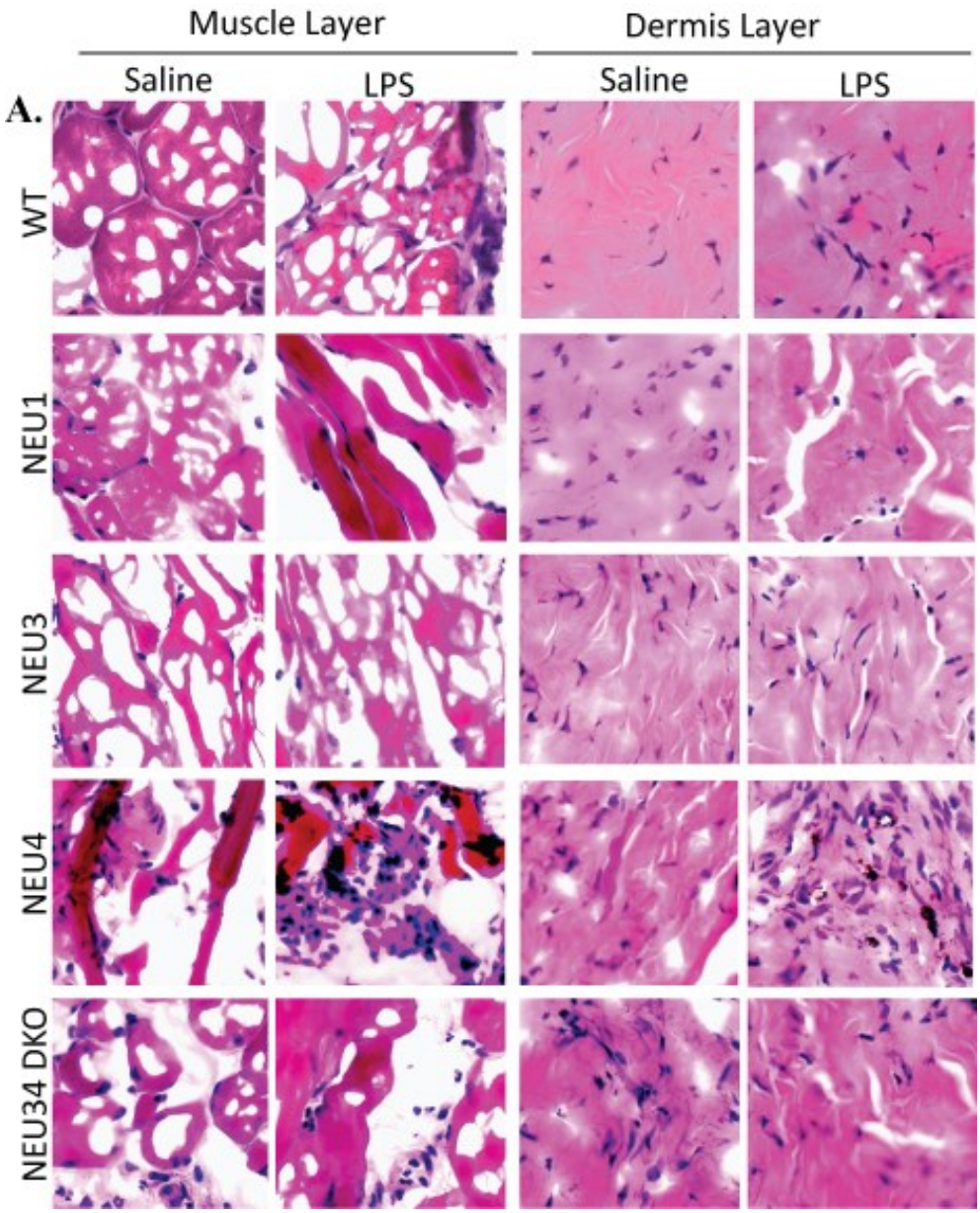


Figure 4.7: Immunohistochemistry of skin slices of the air pouch model. Tissue from the air pouch model was collected, sectioned, and stained (H&E). Regions of tissue were identified as dermis or muscle. Representative images of the dermis and muscle regions of different mice groups. Random fields from each region were used to determine leukocyte counts shown in Fig 4.8.

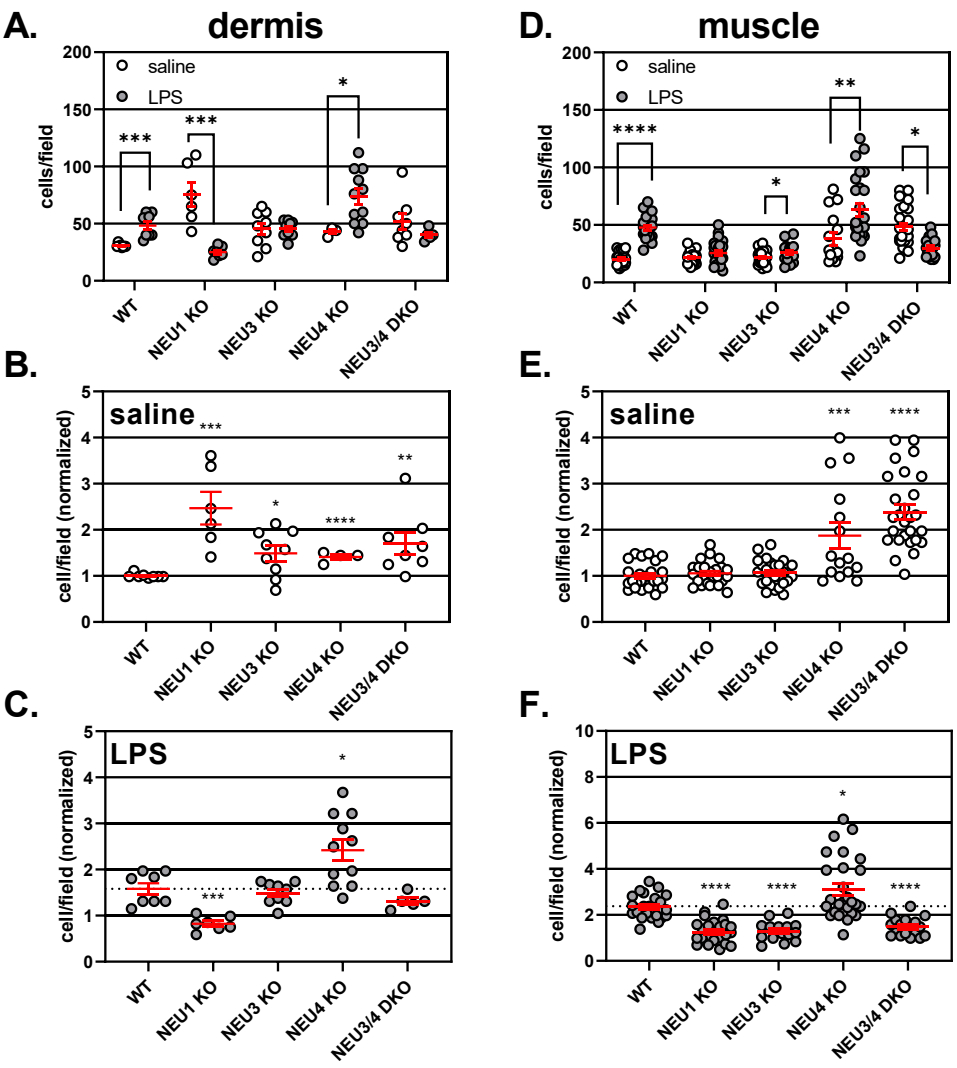


Figure 4.8. Immunohistochemistry of skin slices from the air pouch model. Tissue from the air pouch model was collected, sectioned, and stained (H&E). Regions of tissue were identified as dermis or muscle. See Fig 4.7 for representative images of the dermis and muscle regions of different mice groups. Random fields from each region were used to determine leukocyte counts after saline (○) or LPS (●) treatment. 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 & C) and muscle (E & F) layers, respectively. The data are presented as mean ± SEM, and conditions were compared using a Students t-test (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.005$; ****, $p \leq 0.0001$).

Table 4.7. Leukocyte counts in the air pouch model by IHC.

Muscle layer	Saline (cells/field)		LPS (cells/field)	
	Mean ± SEM	N	Mean ± SEM	N
WT C57BL6	20 ± 1	25	48 ± 3	24
NEU1 KO	21 ± 1	24	26 ± 2	26
NEU3 KO	22 ± 1	27	26 ± 2	17
NEU4 KO	38 ± 6	15	63 ± 5	27
NEU34 DKO	48 ± 3	27	30 ± 2	18

Dermis layer	Saline (/field)		LPS (/field)	
	Mean ± SEM	N	Mean ± SEM	N
WT C57BL6	31 ± 1	8	48 ± 4	8
NEU1 KO	75 ± 11	6	25 ± 2	7
NEU3 KO	45 ± 5	9	45 ± 2	9
NEU4 KO	43 ± 2	4	74 ± 7	11
NEU34 DKO	52 ± 7	8	40 ± 2	5

4.2.6 Neuraminidase enzymes affect macrophage migration *in vitro*

Our observations from the air pouch model of inflammation clearly indicated that NEU isoenzymes can have dramatic effects on leukocyte recruitment to sites of inflammation. To provide insight into a potential mechanism, we investigated the effect of NEU deficiency on the migration of bone-marrow derived macrophages (BDM) from these animals. For the migration assay, cultured BDM from the indicated genotype were seeded into the top chamber of a transwell plate. The lower plate contained a chemoattractant (MCP-1), and the membrane separating the two had been coated with fibronectin (FN). We then counted relative numbers of leukocytes that infiltrated the membrane by microscopy (**Figure 4.9**), the results are plotted in **Figure 4.10**. Previous results have indicated that NEU enzymes modulate the function of integrins, and this experiment set out to test if β1-integrin-mediated migration could be affected by NEU.[29, 30]

The migration experiment revealed that macrophage cells from NEU1 KO animals were not able to transmigrate through a FN matrix. In contrast, macrophage cells from NEU3 KO animals demonstrated increased transmigration into a FN matrix. Macrophages from the NEU4 KO animals showed no differences from WT controls. These results indicate that altered adhesion and transmigration is a potential mechanism for altered leukocyte counts in NEU1 and NEU3 KO animals. The absence of any effects for NEU4 KO animals suggest that a mechanism other than transmigration is involved in the regulation of leukocytes by this isoenzyme.

Table 4.8. Migration of macrophages isolated from different genotypic mice.

	MCP-1 (Cells/field)	
	Mean ± SEM	N
WT C57BL6	8 ± 1	32
NEU1 KO	2 ± 1	19
NEU3 KO	17 ± 1	24
NEU4 KO	6 ± 1	32

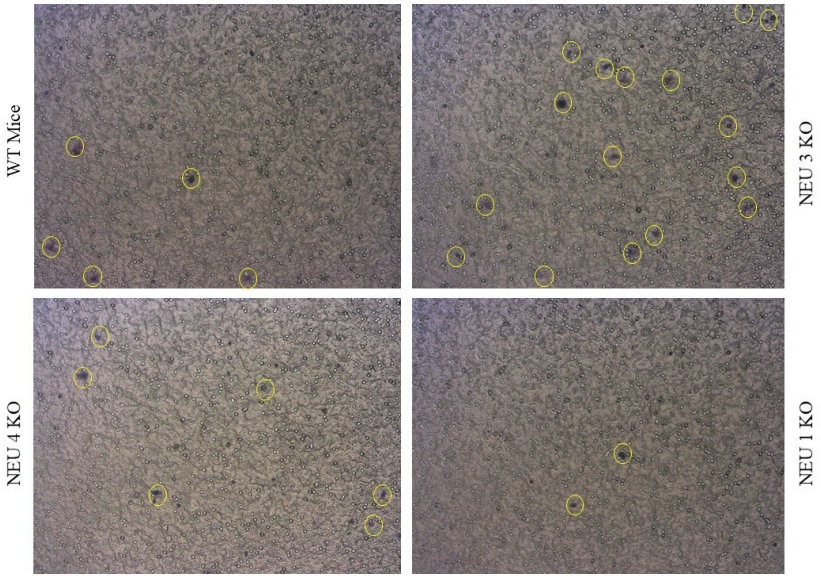


Figure 4.9: Representative images from migration experiments. Macrophages were isolated and differentiated from bone marrow of WT, NEU1 KO, NEU3 KO, and NEU4 KO mice. Cells (2.5 x10^4) were placed in the upper chamber of the transmigration plate and the experiment was carried out for 5 h. The number of cells that infiltrated the FN-coated membrane was determined by counting of stained cells. The number of cells that infiltrated the FN-coated membrane was determined by counting of stained cells. A representative set of brightfield images is shown in the upper panel using a 10 X objective. Cell counts are shown in Fig 6.

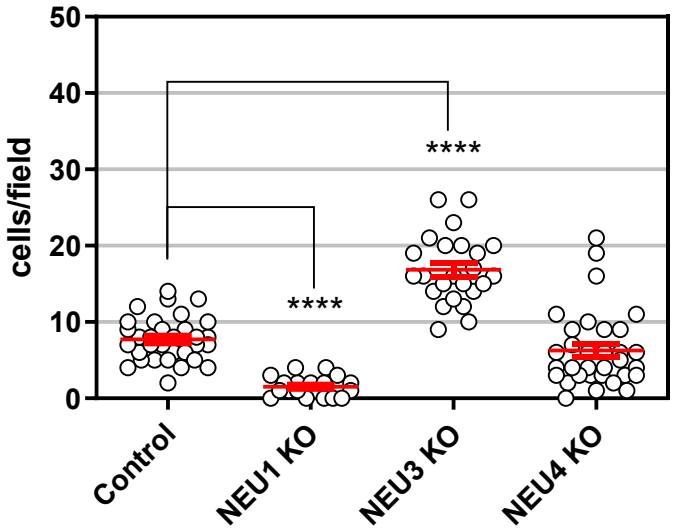


Figure 4.10. Transmigration of bone marrow-derived macrophages is affected by NEU expression. Macrophages were isolated and differentiated from bone marrow of WT, NEU1 KO, NEU3 KO, and NEU4 KO mice. Cells (2.5×10^4) were placed in the upper chamber of the transmigration plate and the experiment was carried out for 5 h with MCP-1 as a chemoattractant in the lower chamber. The number of cells that infiltrated the FN-coated membrane was determined by counting of stained cells. Cell counts are plotted as mean ± SEM. Conditions were compared using a Students t-test (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.005$; ****, $p \leq 0.0001$).

4.3 **Discussion**

Using an air pouch model of inflammation and a unique panel of specific neuraminidase inhibitors, we have investigated the involvement of neuraminidase enzymes in the regulation of acute inflammation in response to LPS. We saw significant differences in the inflammatory response of NEU-deficient mice. In our model of inflammation, NEU1 and NEU3 enzymes were generally pro-inflammatory. In contrast, NEU4 was anti-inflammatory. Thus, reduction of NEU1 and NEU3 activity resulted in reduced leukocyte counts at the site of inflammation; while reduction of NEU4 activity resulted in increased leukocyte counts. Analysis of the leukocyte subsets identified distinct effects of NEU on MO, NE, Mφ, and NK cells. Histochemical analysis of leukocyte infiltration in tissue at the site of inflammation supported the same conclusions. We observed significant modulation of systemic cytokine levels in NEU-deficient animals and speculate that decreases in IL-6 (for NEU1 KO) and increases in IL-21 (for NEU4 KO) are partly responsible for the observed effects on leukocyte recruitment. Finally, we analyzed the transmigration of BDM and observed that NEU1 KO cells were deficient in transmigration, while NEU3 KO cells had enhanced transmigration. This finding strongly suggests that different mechanisms are responsible for each NEU isoenzyme's effect on leukocyte activation.

We designed this study to test the role of NEU in leukocyte response to inflammation directly; however, many previous examples support the involvement of these enzymes in cellular immunity. Neuraminidase enzymes are known to be involved in regulating adherence, spreading, deformability, and function of murine

neutrophils.[11] Endogenous NEU activity on leukocytes can activate cytokines,[31] alter expression of β2 integrin activation epitopes,[10] reduce expression of CD15s,[9] and alter the production of cytokines in response to LPS.[14] NEU1 is involved in the regulation of macrophage phagocytosis.[32] Bacterial neuraminidase treatment has been shown to promote neutrophil, lymphocyte, and macrophage infiltration in rat kidney.[33] NEU1 has been reported to enhance the activities of monocytes and macrophages.[34] Chemical inhibitors of NEU have been shown to modulate β1 integrin-mediated adhesion and migration.[29] Sialylation of immune cells, such as T cells and dendritic cells, is higher in their inactive state.[35,36] Treatment of macrophages with LPS leads to decreased NEU1 activity.[37]

There are several potential mechanisms for regulation of leukocyte infiltration by NEU enzymes. We tested the possibility that altered NEU activity could modify integrin-mediated transmigration and observed that this was true in both NEU1 and NEU3 (**Figure 4.10**). Although we observed differences in cytokine levels in these animals, it is not clear if this is a cause or effect of the differences observed for leukocyte infiltration. It is also possible that NEU-deficient animals respond differently to cytokines or LPS. NEU1 has been shown to be involved in regulation of the mammalian toll-like receptors (TLRs) such as TLR-4,[38,39] which are crucial for immune response to LPS. Furthermore, NEU1 overexpression in human neuroglia and THP-1 cells resulted in the production of IL-6.[40] We hope that future investigations of leukocyte activation will shed more light on the specific mechanisms involved for each of the NEU isoenzymes.

4.4 **Conclusion**

In this study we 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 act as a pro-inflammatory regulators, while NEU4 was strongly anti-inflammatory. Thus, we observed that NEU and NEU3 KO animals showed reduced leukocyte response to LPS, and NEU4 KO animals showed dramatically increased leukocyte response. The effects of NEU isoenzymes on the response of leukocyte subsets showed that MO, NE, Mφ, and NK cells were differentially regulated. For example, NEU1 acted as a positive regulator for these subsets while NEU3 only showed positive regulation of NE. We confirmed these results through immunohistochemistry analysis of dermis and muscle tissue at the site of inflammation and observed similar trends for each enzyme. Systemic cytokine levels in NEU-deficient mice showed several notable differences from WT animals. We noted attenuated levels of IL-6, INFγ, CCL5, and G-CSF in the NEU1 KO, and activation of IL-21 concurrent with attenuation of G-CSF and CCL5 in NEU4 KO animals. Finally, we analyzed the ability of BDM macrophages from NEU-deficient animals to transmigrate in vitro. These experiments showed that NEU1 KO cells had a reduced ability to transmigrate, while NEU3 KO cells had increased transmigration. This experiment suggests that the NEU enzymes may influence multiple mechanisms of regulation in LPS mediated inflammation.

We tested previously developed inhibitors of NEU isoenzymes to confirm the role of enzyme activity in the regulation of leukocytes observed here. Treatment of

WT animals with inhibitors of NEU1 (compound **1**) and NEU3 (compound **2**) reduced the LPS-induced response of multiple leukocyte subsets. Experiments with a NEU4-specific inhibitor (compound **3**) increased LPS-induced response of NE. These results confirm the role of NEU enzyme activity in leukocyte activation and warrant future investigation of these compounds for anti- and pro-inflammatory therapeutics.

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 a new target 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.

4.5 Materials and Methods

4.5.1 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.[41] Mice were housed with continuous access to food and water. The temperature and humidity was maintained as regulations and they were put on a 12-h light/dark cycle. The absence of NEU1, NEU3, NEU4 mice was confirmed by PCR techniques and sialidase activity. The experiment has been approved by the Centre de Recherche/Chu Sainte-Justine (protocol #710). WT and NEU1 KO group contained both male and female mice, while other groups contained males mice only.

4.5.2 Sources of reagents and antibodies

Compounds 1, 2, and 3 were prepared as previously reported.[21-23, 42] Stock solutions for all compounds were made using saline. An LPS isolated from *E. coli* O55:B5 that does not contain sialic acid[43] was purchased from Sigma Aldrich. Anti-mouse/human CD11b (M1/70), anti-mouse CD45 (30-F11), anti-mouse/human CD45R/B220 (RA3-6B2), anti-mouse 49b (Pan-NK cells) (DX5), anti-mouse F4/80 (BM8), anti-mouse Ly-6G (1A8), anti-mouse Ly-6C (HK1.4), and anti-mouse CD115 (CSF-1R) (AFS98) were purchased from Biolegends, USA.

4.5.3 Air pouch model of acute inflammation

Mice (6-8 weeks, male or female) were selected and separated in groups. Hair was removed at the dorsal area (5 cm x 2 cm) two days prior to commencing the experiment. Vaseline was applied on the nude area to alleviate discomfort of the

mice. On day three, the mice were anesthetized under isoflurane. After anesthesia was achieved, 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. Mice were then returned to their cages and checked for restoration of normal functions after recovery from anesthesia. Regular checkup of the mice group was performed to find abnormal behaviors and look for the end point of the study. On day six, the mice were anesthetized again using isoflurane and after anesthesia was achieved, 3 mL of sterilized air (passed through 0.2 μm filter) was injected in the same pouch area made on day-1 using a sterile 26-gauge needle. For treated mice, an intraperitoneal injection was done at this point. Mice were once again returned to their cages and checked for restoration of regular functions after recovery from anesthesia. On day-7, mice were checked for abnormal behaviors, skin injuries and other endpoints.

Mice were again injected intraperitoneally with 200 μL of saline. Mice treated with inhibitors were injected with containing compounds **1**, **2**, or **3** (for a dose of 1 mg/kg). On day-8, mice were checked for abnormal behaviors, skin injuries and other endpoints. Mice were injected intraperitoneally with 200 μL of saline or saline containing inhibitors (1 mg/kg). On day-9, 1 mL of sterile PBS or LPS (1 μg/mL) in sterile PBS was injected into the air pouches. At nine (9) h post-injection, mice were sacrificed using phenobarbital overdose (150 mg/kg bd. wt.) The air pouches were washed with HBSS containing 10 mM EDTA (1 mL, 2X 2 mL). The exudates were collected and centrifuged at 100 x g for 10 min at room temperature. The

supernatants were collected and frozen for later analysis. Cells were re-suspended in 1 mL of HBSS-EDTA and counted by flow cytometry.

4.5.4 FACS analysis of immune cells

To identify cell subpopulations we used FACS of cells isolated from the air pouch exudate based on previously described methods.[44] We used specific fluorophore-conjugated antibodies to sort immune subpopulations based on the following markers: CD49b, CD45, CD115, Ly-6C, Ly-6G, F4/80, B220, CD11b, CD3, and CD11c. Conjugated antibodies (BioLegend, USA) were used as a mixture. Briefly, cells were re-suspended in PBS (50 µL) containing 1 µg human IgG per 10^6 cells and incubated for 15 min at 4 °C to block non-specific binding. Cells were then stained with the mixture of conjugated antibodies (30 min at 4°C) in the dark. Cells were then fixed with 2% PFA and sorted using LSR Fortessa (BD Biosciences, USA), and the data were analyzed by Diva and Flowjo.

4.5.5 Immunohistochemistry protocol and cell counts

Immunohistochemistry (IHC) slides of pouch tissue were prepared by making 4 µm slices and then staining them with hematoxylin and eosin (H&E). The slides were then photographed using a ZEISS Axio Scan Z1. Regions of the IHC slides were identified as three major regions: Epidermis layer, Dermis, and underlying muscle layer. The H&E stain revealed leukocytes present in each region. The number of leukocytes were counted using at least three randomly chosen fields of the same region. Skin sections of at least three mice were investigated from each group.

4.5.6 Cytokine analysis using ProcartaPlex immunoassays

Levels of cytokines in the animals were investigated using bead based immunoassays (ProcartaPlex, eBioscience) with a custom panel according to manufacturer protocol on a Luminex 100 analyzer (Perkin Elmer, USA). Data for cytokine levels of G-CSF, IL-1α, IL-1β, IL-15/IL-15R, IL-21, IL-6, INF-γ, IP-10, and RANTES. We also tested expression of GM-CSF, IL-10, IL-12p40, IL-25/IL-15, MCP-1, MIP-1α, MIP-1β, and MIP-2. However, the expression level difference for these cytokines were unremarkable and values were not shown. Cytokines were measured in the serum sample collected post experiment.

Cell detected	Antigen	Flurophore	Clone	Isotype	Cat. No.	Company
Pan-NK cells	CD49b	APC	DX5	Rat IgM, κ	108909	Biolegend
All hematopoietic cells	CD45	FITC	30-F11	Rat IgG2b, κ	103107	Biolegend
Monocytes	CD115	PECy 7	AFS98	Rat IgG2a, κ	135523	Biolegend
Monocytes	Ly 6 C	BV421	HK1.4	Rat IgG2a, κ	128031	Biolegend
Neutrophils	Ly-6G	APC/Cy7	1A8	Rat IgG2a, κ	127623	Biolegend
Macrophages	F4/80	PE	BM8	Rat IgG2a, κ	123109	Biolegend
B cells	B220	PerCP	RA3-6B2	Rat IgG2a, κ	103233	Biolegend
Neutrophils, Monocytes, Eosinophiles	CD11b	eVolve 605	M1/70	Rat / IgG2b, kappa	83-0112-41	Invitrogen

4.5.7 Macrophage isolation and chemotaxis trans-migration studies

Monocytes were isolated from the bone marrow of mice after differentiation to macrophages. The macrophages were cultured for 5 days in a T75 flask when they reached 80% confluency. Cells were then used for a transmigration assay adopted

from a previous study.[45] Briefly, an 8-µm-pore size transwell migration plate (Costar, Fischer Sci, USA) was used after the transwell membrane was coated with 50 µg/mL fibronectin (FN) in PBS for 1 h and blocked with bovine serum albumin (BSA) for 30 min. The bottom chamber of the plate contained PBS with 20 ng/well monocyte chemoattractant protein-1 (MCP-1, Thermofisher Scientific, USA) as the chemoattractant. Macrophages ($5x10^4$ cells in PBS) isolated from the genotypic mice were carefully placed on the top chamber and incubated for 5 h at 37 °C with 5% CO_2. Subsequently, the inner top side of the membrane was carefully wiped using a Q-tip and the transwell membrane was stained using diff quik stain (Fischer, USA). Cells that had infiltrated the membrane were then imaged using Zeiss COLIBRI fast LED imaging and optical sectioning microscope using 10X lens under brightfield. The images were analyzed using Zen black software.

4.6 **References**

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5. Conclusions and future directions

5.1 **Overview**

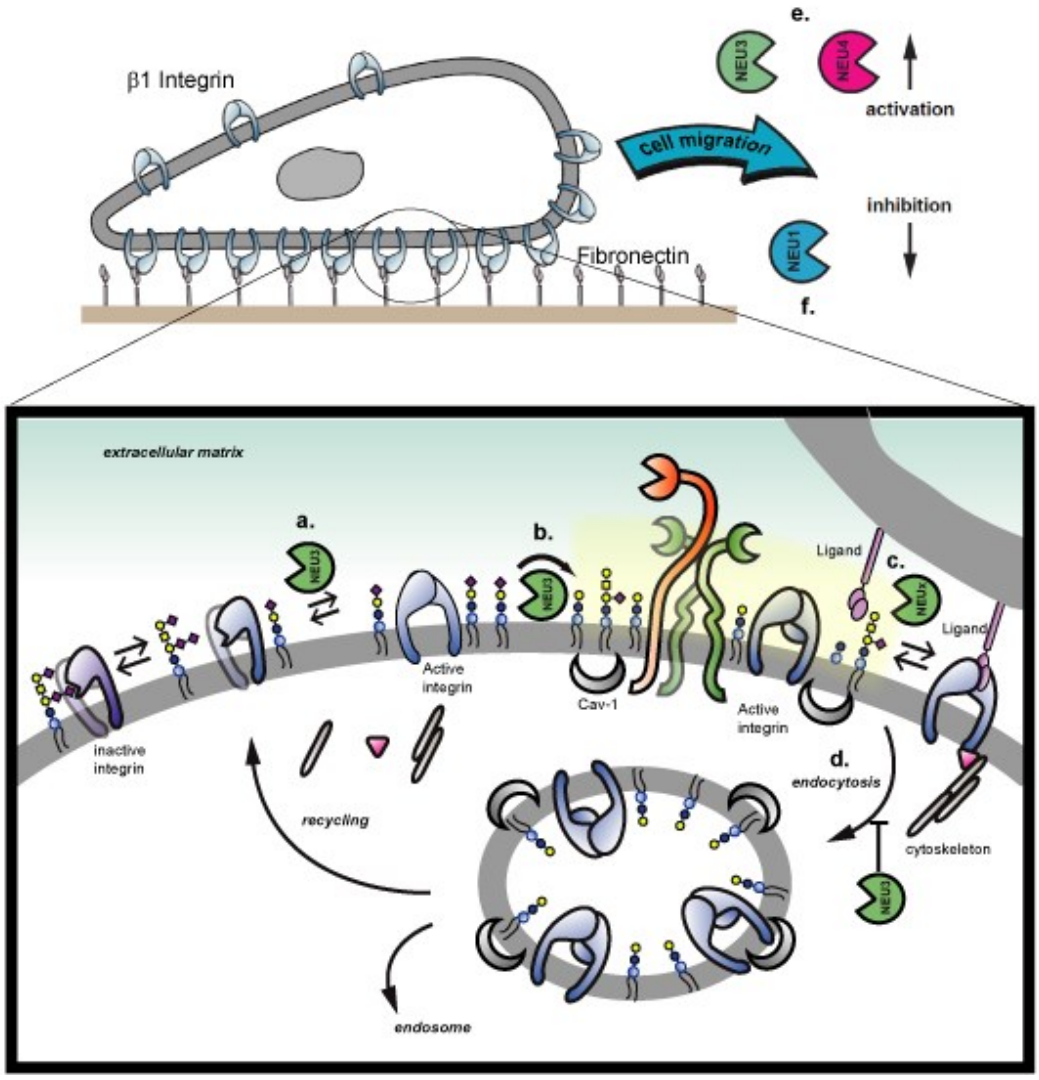

Figure 5.1. Overview of this thesis. (a) NEU3 was found to be involved in integrin activation. (b) NEU3 showed glycolipid modification leading to modification of integrin functions (c) NEU3 has been shown to be a negative regulator of integrin ligand binding such as LFA1-ICAM1 interactions. (d) NEU3 has been shown to be involved in integrin endocytosis.(e) NEU3, and NEU4 has been shown to be activator of cell migration.(d) NEU1 as found to be an inhibitor of cell migration.

In Chapter 2, we found that NEU3 modified the lateral mobility of LFA-1, which is a corner stone of leukocyte adhesion and transmigration. We also showed NEU3 to be an important regulator of the adhesion properties of LFA-1 to ICAM-1 *in vitro* and in the endocytosis of the LFA-1 molecule. These studies are consistent with hNEU involvement in inflammation, and suggest that inhibitors of hNEU could be potential anti-inflammatory therapeutics.

In Chapter 3, we focused on the effect of hNEU isoenzymes on β1-integrin mediated cell migration. Inhibition of NEU1 seemed to be pro-migratory, and NEU3 and NEU4 isoenzymes were anti-migratory.

In Chapter 4, we used an in vivo model to demonstrate the effect of hNEU enzymes in LPS-induced acute inflammation. In mice, leukocyte infiltration into inflamed site was sensitive to mNEU isoenzymes.

Together, the experiments presented in this thesis indicate that there is substantial involvement of neuraminidase isoenzymes in activation and infiltration of leukocytes and the migration of cells. Although further studies will be required to understand the mechanisms of this regulations, these findings highlight a previously unknown role of neuraminidase enzymes in immune cell regulation.

5.1 **Future directions**

We observed that human neuraminidase enzymes are involved in cell migration (Chapter 3), cell adhesion (Chapter 2), and inflammation (Chapter 4). However, there are many other important aspects of hNEU regulation that remains to be

answered. We summarize some specific questions that may deserve future investigation below.

5.1.1 Effect of neuraminidase isoenzymes in regulation of integrin activation

Integrins are major players in cellular functions like adhesion, interaction and cell migration. They mainly exist in multiple conformational states.[1,2] Integrins cycle between these conformations and become active through "inside-out" signaling process triggered through G-protein coupled receptors, chemokines, or cytokines.[3] In Chapters 2 & 3 we focused on the importance of neuraminidase enzymes in the modulation of neuraminidase enzymes, but their effect on the activation of integrins have been largely unexplored.

Methods to investigate the effects of hNEU on integrins could include single-cell FRET assays. Additionally, flow-cytometry based protocols where individual cells can be observed for their activation state could provide a population-based measurement of this phenomenon. A similar method has been described by Sambrano et. al.[4]

5.1.2 Identify the specific biological substrates of neuraminidase isoenzymes

Human neuraminidase enzymes hydrolyze sialic acid from glycoproteins and glycolipids. Previous experiments have attempted to isolate and characterize the substrates for the each of the four neuraminidase enzymes.[5] Although several glycolipid substrates have been defined for NEU isoenzymes, characterization of specific glycoprotein substrates remains largely unknown. This is most likely due to the complexity of glycoprotein substrates and the difficulty in quantitative

analysis of these biomolecules. To understand the effect of NEU on post-transitional modifications in biological processes, specific substrate targets of the NEU isoenzymes need to be identified. Furthermore, these targets must be resolved as specific or non-specific substrates. Methods to address this question would likely rely on affinity enrichment of sialoside-containing proteins and mass spectrometry or proteomics.

5.1.3 Develop robust assays and diagnostic tools to detect activity of hNEU isoenzymes

Analysis of different isoenzyme activity is crucial in elucidation of their functions in vivo. Currently, most groups involved in the investigation of NEU use the fluorogenic substrate 4MU-NANA as their primary tool to measure enzyme activity. The 4MU-NANA probe has little specificity for individual isoenzymes, and therefore does not provide resolved activity data. Using 4MU-NANA in a cell-based assay to report neuraminidase activity may provide confounding results, as the probe will report on the activity of multiple isoenzymes as well as any contaminating enzyme (e.g. hNEU).

One potential strategy is to use the 4MU-NANA assay in the presence of high concentrations of selective hNEU inhibitors. For example, testing cell lysate with 4MU-NANA in the presence or absence of NEU3 and NEU4 inhibitors[6] can provide insight into the isoenzymes which are most active in the sample.

Perhaps the most widely used method for the study of neuraminidase isoenzymes is the genetic manipulation of these genes in animal models. This strategy is not ideal for understanding canonical biological processes, and may present challenges for making direct comparisons.

Development of hNEU isoenzyme-specific substrates could be used to develop diagnostic assays. Alternatively, validated antibodies against the hNEU isoenzymes might allow for quantitative determinations of protein levels, though they may not report on enzyme activity.

5.1.4 Investigate the role of neuraminidase enzymes specific endocytic pathways

In Chapters 2 & 3, we proposed that hNEU may influence integrins through changes in endocytosis. However, there are multiple well-known endocytic pathways that contribute in the endocytosis of integrins. These pathways need to be investigated further to determine the influence of the hNEU isoenzymes on these processes. The regulation of endocytosis could make hNEU a valuable therapeutic target. Diseases that involve misregulation of endocytosis such as mucopolysaccharidosis,[7] Fabry diseases (α-galactosidase A deficiency),[8] Pompe diseases (acid α-glucosidase deficiency),[9] centronuclear myopathy,[10] Charcot-Marie-Tooth,[10] Chediak Higashi syndrome,[11] Hermansky-Pudlak syndrome,[11] Dannon diseases, and others, may have pathophysiology related to hNEU enzymes. Thus, defining the role of hNEU in these processes could provide insight to important disease targets.

The effect of neuraminidase enzymes in the metabolism of insulin by adipose tissues has been known since 1970.[12] Recently, positive regulation of insulin signaling by IR has been proposed for NEU1.[13] Furthermore, activation of NEU1 expression by ambroxol (an activator of lysosomal gene network) was able to reverse the insulin resistance in obese mice.[6] Insulin is a hormone secreted from the

pancreas which needs to be internalized in cells to function. Interestingly, NEU3 has also been proposed to be a regulator of insulin signaling and overexpression of NEU3 has been shown to be beneficial in increasing insulin sensitivity and glucose tolerance.[14] The effect of NEU4 on insulin uptake and glucose metabolism remains largely unknown.

Opposite effects of NEU1 in insulin regulation has also been reported where NEU1 reduces insulin receptor signaling.[15] Chronic overexpression of NEU1 in mice produced insulin resistance and development of diabetic phenomenon.[6] These results may suggest a complex mechanism regulated by the neuraminidase enzymes.

Insulin receptors are critical for insulin signaling and insulin receptor undergoes caveolin-mediated endocytosis.[16] NEU3 has also been associated with caveolin in membrane microdomains,[17] and shown to regulate caveolin-mediated endocytosis of β1 integrin.[18] The relationship of NEU1 and NEU4 to IR is still not well understood. A focused study on hNEU isoenzyme effects on caveolin-mediated endocytosis might give us more insight on the insulin signaling and could be used to develop a novel therapeutic pathway for diabetic therapies.

5.1.5 Investigate the role of specific hNEU inhibitors in diseases

Possible regulation of arthritis by neuraminidase enzymes has been reported in arthritic rats in 1978.[19] A recent pilot study found very high correlation between the levels of NEU3 in B cells of patients suffering from rheumatoid arthritis (RA).[20] A correlation between NEU3 expression levels in monocytes of rheumatoid arthritis patients was observed. However, effects of NEU1 and NEU4 was not

investigated. Further validation is required to understand the effect of hNEU isoenzymes in the pathology of rheumatoid arthritis.

Possible regulation of programmed cell phagocytosis (PrCP) and neuraminidase has been proposed by Feng et. al.[21] They observed that NEU4 KO cells had decreased the PrCP of tumor cells. However, the effects of the NEU1, NEU2, and NEU3 isoenzymes remains unknown and needs to be tested further.

A disease model for RA can be generated in bone marrow-derived macrophages of different NEU isoenzymes KO mice and measure their uptake of bacteria (stained), which might be useful in understanding the function of each neuraminidase isoenzymes.

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