The role of human neuraminidases in LFA-1–ICAM-1 adhesion

by

Yue Fan

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Department of Chemistry
University of Alberta

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Abstract

The *N*-acetyl neuraminic acid (sialic acid) residue is a crucial monosaccharide in mammalian glycosylation and commonly serves as the terminal residue on glycoproteins and glycolipids. As a key regulation site in glycosylation, it plays an essential role in modulating cell-cell interactions and signaling. Human neuraminidase (hNEU) enzyme modulates glycosylation by cleaving the terminal sialic acid residues from glycoconjugates and are involved in various biological processes. The hNEU family comprises four distinct isoenzyme members (NEU1, NEU2, NEU3, and NEU4) with diverse functions. For instance, NEU1 is primarily localized in lysosomes and degrades glycoconjugates, while NEU3 is mostly found on the plasma membrane and modifies glycolipid substrates.

Cells of the immune system use glycoprotein receptor systems to engage in specific cell-cell interactions. Many immune receptors are heavily glycosylated, and the role of sialic acid in these interactions has not been well characterized. To test if hNEU has a role in regulation of leukocyte adhesion we investigated individual hNEU isoenzyme effects on a critical adhesion system of leukocytes. We implemented an in vitro assay for LFA-1–mediated cell adhesion using flow cytometry. We observed that cells pre-treated with selective inhibitors of hNEU had increased LFA-1 adhesion. Additional controls support that binding is specific, and that native hNEU isoenzymes act as negative regulators of LFA-1 adhesion in leukocytes during inflammation. While hNEU3 has a selective effect on static adhesion, hNEU1 and hNEU4 regulate both static

and activated states. These results suggest that targeting specific hNEU isoenzymes could serve as a potential therapeutic strategy for anti-inflammation treatments.

In Chapter III, we investigated development of an assay to detect the activity of individual hNEU isoenzymes in human blood. To achieve this, we developed a direct detection assay by using a standard 4MU-NANA assay with and without hNEU selective inhibitors, allowing for measurement of each isoenzyme activity.

Preface

The work in Chapter II was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC, RGPIN 2020-04371) and the National Institutes of Health (R01-HL153264). Inhibitors used in the study were prepared by Elisa Garcia Carvajal and Mostafa Radwan (Prof. C. Cairo group, U Alberta). The lectin staining compound diCBM40-Alexa Fluor647 was provided by Prof. L. Mahal (U Alberta). Prof. J Acker and Sanaz Hemmatibardehshahi (U Alberta) assisted with donor sample collection. We thank Chuanhao Peng and Chih-Lan Lin (U Alberta) for helpful discussion. A version of Chapter II is currently under review for publication.

In Chapter III, serum and plasma samples from healthy donors were provided by the West laboratory (University of Alberta). Inhibitors used in this study were prepared by Elisa Garcia Carvajal and Mostafa Radwan (Prof. C. Cairo group, U Alberta).

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List of abbreviations

AIDS	acquired immunodeficiency syndrome
Asn	asparagine
CBM	carbohydrate-binding modules
CD	cluster of differentiation
Cosmc	core 1 β3-galactosyltransferase-specific molecular chaperone
CR	complement receptor
CTLD	Calcium dependent lectin domain
DANA	2-deoxy-2,3-didehydro-*N*-acetyl neuraminic acid
EGF	epidermal growth factor
ER	endoplasmic reticulum
GSL	glycosphingolipids
hNEU	human neuraminidase
ICAM-1	intercellular cell adhesion molecule-1
KO	knock-out
Lac-Cer	lactosyl-ceramide
LAD-1	leukocyte adhesion deficiency-1
LeX	Lewis X
LFA-1	lymphocyte function-associated antigen 1
LPS	lipopolysaccharide
MAC-1	macrophage-1 antigen
NEU	neuraminidase

Neu5Ac	*N*-acetylneuraminic acid/sialic acid
NS	not significant
OC	oseltamivir carboxylate
PBMC	peripheral blood mononuclear cell
PNA	peanut agglutinin
PSGL-1	P-selectin glycoprotein ligand-1
RT	room temperature
SCID	severe combined immunodeficiency
Ser	serine
sLeX	sialyl Lewis X
SNA	sambucus nigra agglutinin
ST	sialyl-transferase
Thr	threonine
VCAM-1	vascular cell adhesion molecule-1
VLA-4	very late antigen-4
WT	wild-type
ZAN	zanamivir

Chapter I

The roles of hNEU in inflammatory response and activity in human serum

1.1 Immune system function is essential for maintenance of health and resolution of inflammation

The immune system is a critically important and indispensable component of the human body. The immune system plays a crucial role in defending and protecting the body from ever present bacteria, viruses, and other pathogens in the surrounding environment. Each component of the immune system has its specific function, working to maintain the integrity of immunological function. When immune function is defective, it can lead to acute diseases. Disruption of the immune system results in life threatening diseases such as: severe combined immunodeficiency (SCID; a genetic immunodeficiency disorder where patients cannot survive outside of a sterile environment)[1] and acquired immunodeficiency syndrome (AIDS; caused by human immunodeficiency virus). These diseases result in immune dysfunction and can lead to severe complications. These examples highlight the essential role of the immune system in human health.

One of the key components of the immune system is its ability to initiate and regulate inflammation, which serves as a defense mechanism against harmful challenges such as infection, injury, or toxins.[2] Inflammation typically starts as an acute response, but if it is not properly controlled or persists over time, it can transition into a chronic state.[3] Chronic inflammation is associated with a variety of serious health conditions, including cardiovascular disease, atherosclerosis, obesity, diabetes, and cancer.[4,5] Therefore, managing inflammation is crucial because prolonged or uncontrolled inflammation can not only exacerbate existing conditions, but also contribute to the

development of new diseases.[5] Maintaining a balanced immune response and controlling inflammation are essential for preventing long-term health complications and ensuring overall health.[6]

1.1.1 Leukocytes: types and functions of immune cells

A critical aspect of the inflammatory response is the recruitment of immune cells to the site of injury or infection.[7] Leukocytes (or white blood cells) are central to the inflammatory response. Leukocytes are composed of many cell types including T-cells, B-cells, macrophages, neutrophils, and platelets, each with distinct functions in immune response. For instance, T cells, which mature in the thymus, are classified into three main types: helper T cells (assist other immune cells), cytotoxic T cells (destroy infected or cancerous cells), and regulatory T cells (maintain immune balance).[8] B cells are responsible for recognizing antigens and producing antibodies as part of humoral immunity.[8] Together, T cells and B cells are referred to as lymphocytes, a key subset of leukocytes. Macrophages, as their name implies, are large phagocytic cells that phagocytose and digest pathogens.[8] Neutrophils, the most abundant type of white blood cell, directly fight and kill pathogens through phagocytosis.[8] Lastly, platelets (although not true cells) are anuclear cell fragments that circulate in the blood and play a dominant role in hemostasis by forming clots at injury sites.

Leukocyte Cell Type	Major Adhesion Receptor(s)	Ligand(s)
Lymphocytes (T cells, B cells)	LFA-1($\alpha_L\beta_2$, CD11a/CD18) VLA-4($\alpha_4\beta_1$, CD49d/CD29) L-selectin(CD62L) PSGL-1(CD162)	ICAM-1(CD54) VCAM-1(CD106) PSGL-1(low affinity) E/P-selectin(CD62E/P)
Macrophages	LFA-1($\alpha_L\beta_2$, CD11a/CD18) MAC-1(CR3, $\alpha_M\beta_2$, CD11b/CD18) CR4($\alpha_X\beta_2$, CD11c/CD18) PSGL-1(CD162)	ICAM-1(CD54) ICAM-1(CD54) ICAM-1(CD54) E/P-selectin(CD62E/P)
Neutrophils	LFA-1($\alpha_L\beta_2$, CD11a/CD18) MAC-1(CR3, $\alpha_M\beta_2$, CD11b/CD18) CR4($\alpha_X\beta_2$, CD11c/CD18) L-selectin(CD62L) PSGL-1(CD162)	ICAM-1(CD54) ICAM-1(CD54) ICAM-1(CD54) PSGL-1(low affinity) E/P-selectin(CD62E/P)
Platelets	P-selectin(CD62P)	PSGL-1

Table 1.1: Cell types of leukocytes and their adhesion receptors.

Adapted from 1. Murphy, K.; Weaver, C. *Janeway's Immunobiology*, 9th edition; Garland Science/Taylor & Francis Group, LLC: New York, NY, 2016. 2. Ley, K.; Laudanna, C.; Cybulsky, M. I.; Nourshargh, S. Getting to the Site of Inflammation: The Leukocyte Adhesion Cascade Updated. *Nat. Rev. Immunol.* **2007**, *7* (9), 678–689.

Immune cells, like all cell types, express specific receptors on their surfaces that may be used for binding or interacting with other cells. Common receptors have been identified using antibodies and are organized by cluster of differentiation (CD) by the HLDA workshops. These markers can then be used to identify proteins or glycoproteins on the cell surface.[9] Leukocyte cell surface receptors play essential roles in biological processes such as immune response, cell signaling, adhesion, and migration (see **Table 1.1**).[8] Among the important adhesion receptors on leukocytes, CD18 is critical for mediating cell adhesion and migration, particularly during inflammatory responses. CD18, also known as the β_2 integrin, pairs with different α subunits to form heterodimers, such as $\alpha_L\beta_2$ (LFA-1), $\alpha_M\beta_2$ (MAC-1), and $\alpha_X\beta_2$ (CR4), which are essential for leukocyte adhesion and recruitment. In individuals with genetic variations in the gene encoding CD18, the resulting absence of adhesion receptors leads to leukocyte adhesion deficiency-1 (LAD-1).[10,11] As the name implied, LAD-1 results in defective leukocyte adhesion, preventing leukocytes from reaching infection sites to eliminate pathogens. This condition mimics the immunodeficiency observed in SCID, causing affected individuals highly susceptible to infections.

1.1.2 Essential adhesion receptors in the inflammatory process

The immune cells in human blood play a central role in the inflammatory process. When inflammation is initiated, the infected site releases specific inflammatory signals which can trigger a signaling cascade inducing leukocyte recruitment.[12] The recruitment process includes multiple steps that activate the cells, and allow them to traverse the endothelial barrier, and reach the site of inflammation. Central to each step in this

process are adhesion receptors, cell surface molecules that specifically recognize their ligands allowing the initiation, maintenance, and release of cell-cell contacts. This process is carefully orchestrated and involves a complex network of signaling pathways and over 900 proteins.[13] Due to the complexity of leukocyte adhesion, many pathways remain incompletely characterized. The process of leukocyte recruitment is vital for the immune system to respond effectively to threats, aiding in the clearance of infections and the promotion of tissue repair.[14] Therefore, developing an improved understanding of the leukocyte adhesion cascade remains an important endeavor as these processes may be critical in disease or could be important for the development of future therapeutics.

Leukocytes exploit a range of adhesion receptors at different steps of the adhesion cascade. Important receptor families include selectins, integrins, and their ligands that involved in the processes of capture/tethering, rolling, arrest/firm adhesion, and extravasation in response to inflammation signals, as shown in **Figure 1.1**.[13] Selectins are C-type (Calcium dependent) lectins, which are single-chain transmembrane glycoproteins characterized by a Calcium dependent lectin domain (CTLD), an epidermal growth factor (EGF)-like domain, short consensus repeat domains, as well as transmembrane and cytoplasmic domains (in **Figure 1.2a**).[15,16] Selectins are classified as CD62, and mediate cell-cell adhesion, particularly in leukocyte rolling. The selectin family comprises three main members: L-selectin (CD62L), E-selectin (CD62E), and P-selectin (CD62P). Their primary ligand, P-selectin glycoprotein ligand-1 (PSGL-1) is expressed on leukocytes and contains sulfated tyrosine residues and sialyl Lewis X (sLeX), which are specifically recognized and

bound by the CTLD of selectins (**Figure 1.2b**).[16,17] E-selectin and P-selectin are expressed on activated endothelial cells, and their interaction with PSGL-1 facilitates leukocyte rolling on the endothelium. In contrast, L-selectin is expressed on the surface of leukocytes and can also form bonds with PSGL-1. However, these bonds are short-lived, which prevents the aggregation of adjacent leukocytes in blood vessels.[15]

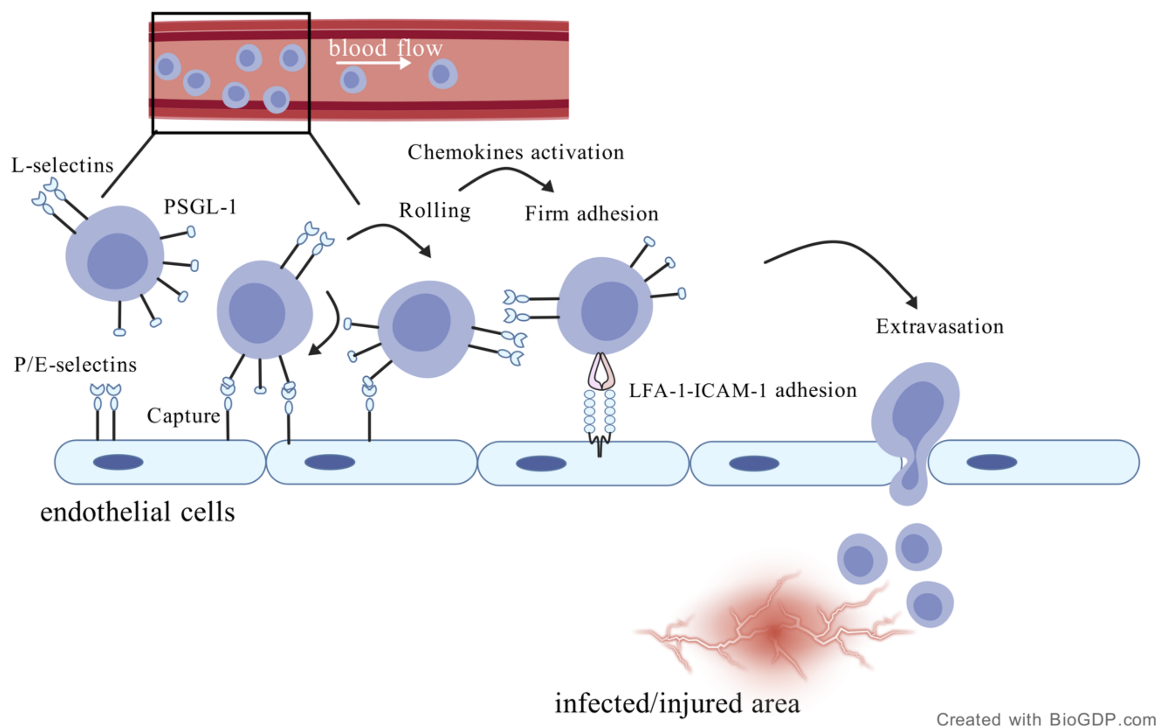

Figure 1.1: Leukocyte adhesion cascade and relevant receptors-ligands.

This figure illustrates the key steps of leukocyte adhesion cascade, including capture, rolling, arrest/firm adhesion, and later phases extravasation. It highlights the specific receptors and ligands involved at each step, such as selectins, integrins, and their respective ligands. Adapted from 1. Ley, K.; Laudanna, C.; Cybulsky, M. I.; Nourshargh, S. Getting to the Site of Inflammation: The Leukocyte Adhesion Cascade

Updated. *Nat. Rev. Immunol.* **2007**, *7* (9), 678–689. 2. Cummings, R. D.; Chiffoleau, E.; Van Kooyk, Y.; McEver, R. P. Chapter34 C-Type Lectins. In *Essentials of glycobiology*; Elsevier, 2010; Vol. 479, pp 223–241. (Figure was created with BioGDP.com)[18]

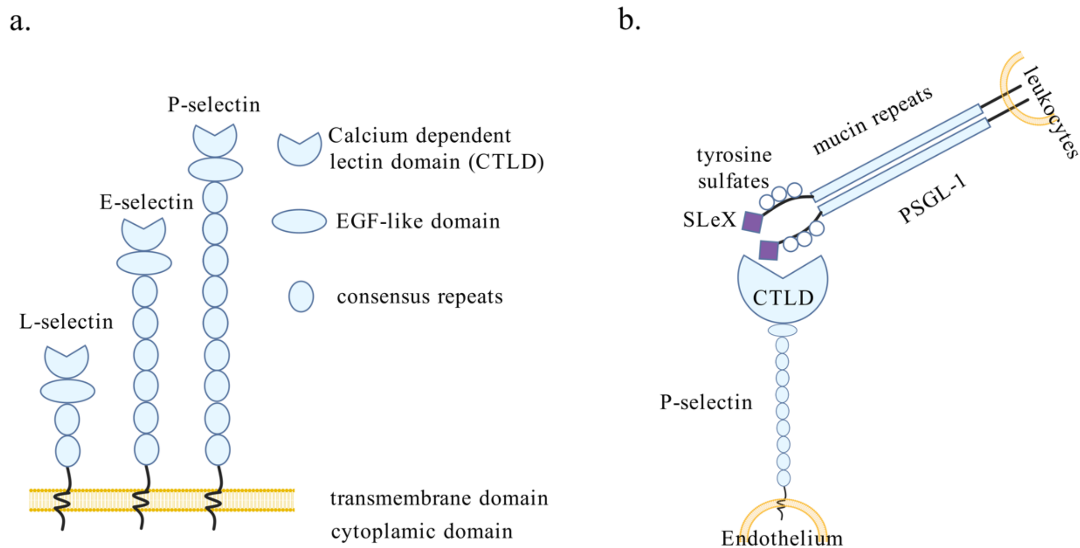

Figure 1.2: The structures of selectins and PSGL-1.

(**a**). The structural domains of the three selectin types: L-selectin, E-selectin, and P-selectins, such as CTLD, EGF-like domain, consensus repeats, transmembrane domain, and cytoplasmic domain. The main differences between the selectins are the number of consensus repeats. (**b**). The structure of PSGL-1, including mucin repeats, N-terminal region with sulfated tyrosine residues and sLeX O-glycans, which are recognized by CTLD of selectins. Adapted from Cummings, R. D.; Chiffoleau, E.; Van Kooyk, Y.; McEver, R. P. Chapter34 C-Type Lectins. In *Essentials of glycobiology*; Elsevier, 2010; Vol. 479, pp 223–241. (Figure was created with BioGDP.com)[18]

Integrins are another class major adhesion receptors found on leukocytes. Integrins are transmembrane glycoproteins that feature N-link glycosylation and consist of α and β subunits forming heterodimers (**Figure 1.3**). As previously discussed in the context of the CD system, β integrins are indispensable for cell interactions and migration, mediating inflammatory responses including the leukocyte adhesion cascade. For instance, β_1 integrins pair with the α_4 subunit to form the very late antigen-4 (VLA-4), which binds to vascular cell adhesion molecule-1 (VCAM-1) and participates in both leukocyte rolling and firm adhesion.[13] Additionally, β_2 integrins can pair with different α subunits to form heterodimers that mediate distinct steps in the inflammatory cascade. For example, $\alpha_L\beta_2$ is the first integrin to participate in firm adhesion, while $\alpha_M\beta_2$ is involved in later phases, such as crawling.[13] These examples highlight the critical roles of adhesion receptors in regulating inflammatory responses, with selectins and integrins coordinating the recruitment and migration of leukocytes during inflammation.

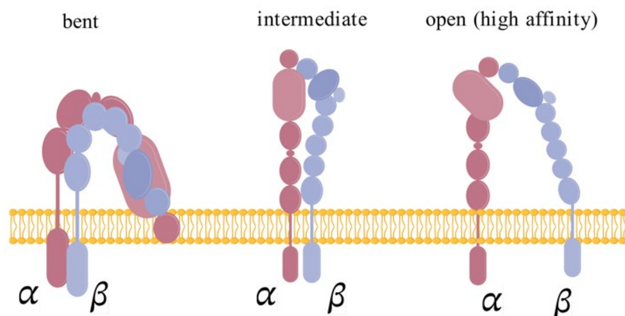

Figure 1.3: The structure and conformational states of integrins.

The β_2 integrin family consists of α (with an αI domain) and β subunits, forming heterodimers such as $\alpha_L\beta_2$, $\alpha_M\beta_2$, and $\alpha_X\beta_2$.[19] Integrins exist in three distinct conformational states: a bent (closed) conformation (left) represents the resting state, and can be induced to an intermediate state (middle) through outside-in signaling, like ligand binding, and then be activated into a high affinity state (right). Adapted from Kadry, Y. A.; Calderwood, D. A. Chapter 22: Structural and Signaling Functions of Integrins. In *Biochimica et Biophysica Acta (BBA) - Biomembranes*; 2020; Vol. 1862, p 183206. (Figure was created with BioGDP.com)[18]

1.2 The role of LFA-1 in leukocyte adhesion cascade

1.2.1 Leukocyte rolling and Selectins

Leukocyte tethering and rolling are mediated by selectins and selectin ligands. This system includes the L-selectin and PSGL-1 on leukocytes, and E-selectin and P-selectin receptors on activated endothelial cells.[20] The interactions between selectins and their ligands are weak, which enables bonds to break and reform leading to a process known as 'leukocyte rolling' on endothelium. The selectin receptor bonds are stabilized and strengthened by the application of shear stress induced by blood flow.[21] This process ultimately results in a process that slows leukocyte traversal in the blood stream, allowing for other adhesion processes to take over which require longer contact times between cells.

1.2.2 Leukocyte capture and firm adhesion

Just as in leukocyte rolling, chemokines and chemoattractants activate integrin receptors, enhancing their avidity to rapidly arrest these leukocytes once they have been captured by leukocyte rolling.[13] As depicted in **Figure 1.4**, avidity refers to the redistribution and clustering of integrins on the cell surface, forming multiple binding sites, while affinity reflects changes at a single binding site, often resulting from confirmational shifts in the integrin.[22] The lymphocyte function-associated antigen 1 (LFA-1) is a β2-integrin which is expressed exclusively on leukocytes, and which is indispensable for leukocyte firm adhesion. It is a heterodimeric glycoprotein composed of αL (CD11a) and β2 (CD18) subunits. The ligand for LFA-1 is intercellular adhesion molecule 1 (ICAM-1, CD54) which has five extracellular immunoglobulin domains, one transmembrane domain, and one cytoplasmic domain.[23] The ICAM-1 receptor is a glycoprotein expressed on activated endothelial cells and leukocytes.[11]

The strength of the LFA-1–ICAM-1 binding interaction is dependent on receptor conformation. As shown in **Figure 1.3**, LFA-1 exists in a bent conformation in its resting state, which can be induced to an intermediate state and subsequently to an open, high-affinity state upon activation.[24,25] In its high-affinity state, LFA-1 forms firm adhesion with ICAM-1 on endothelial cells, arresting leukocytes, and promoting leukocyte extravasation from blood vessel to inflammation sites.[13,23,24] Interestingly, there are a number of monoclonal antibodies that have been identified which recognize specific conformational states of the LFA-1 receptor.[26] Therefore, modulating LFA-1 conformation and affinity, represents a promising therapeutic strategy for anti-inflammatory and other therapeutic strategies.[23,27]

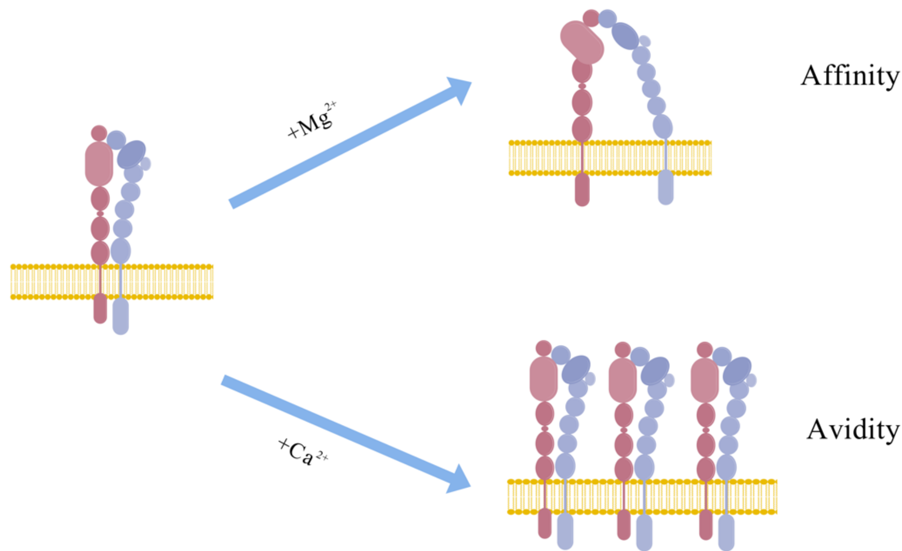

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Figure 1.4: Comparison of affinity and avidity of LFA-1.

For LFA-1, affinity represents Magnesium-induced active conformational changes that enhance the binding strength of individual LFA-1 molecules to ICAM-1. In contrast, avidity emphasizes the redistribution and clustering of LFA-1 molecules on the cell surface, the process stabilized by Calcium which maintains LFA-1 in its inactive state. Adapted from van Kooyk, Y.; Figdor, C. G. Avidity Regulation of Integrins: The Driving Force in Leukocyte Adhesion. *Cell Biol.* **2000**, *12*, 542–547. (Figure was created with BioGDP.com)[18]

1.3 Glycosylation regulation and its impact on inflammation

1.3.1 Major forms of glycosylation in human proteins

Proteins and lipids in human cells may be modified by glycosylation (in **Figure 1.5**), and the specific glycans attached to these glycoconjugates may be important to their recognition and function. The post-translational modification of proteins can take multiple forms, which may include N-link or O-link glycans. Proteins with N-link glycans are modified at asparagine residues (Asn, N), and are linked through the *N*γ atom of the residue to the glycan. In contrast, O-link glycans are linked through Ser or Thr residues as an *O*-glycoside. The structures of these two classes of PTMs differ in their core structures. The N-link glycans are often comprised of hybrid or complex biantennary structures, while O-link glycans contain a set of common smaller core structures. Glycolipids in humans typically consist of a lactosyl-ceramide core structure (Lac-Cer) which is elaborated with shorter glycan chains than the N-link structures. In all cases, these glycans commonly display neuraminic acid (Neu5Ac) residues at their termini.

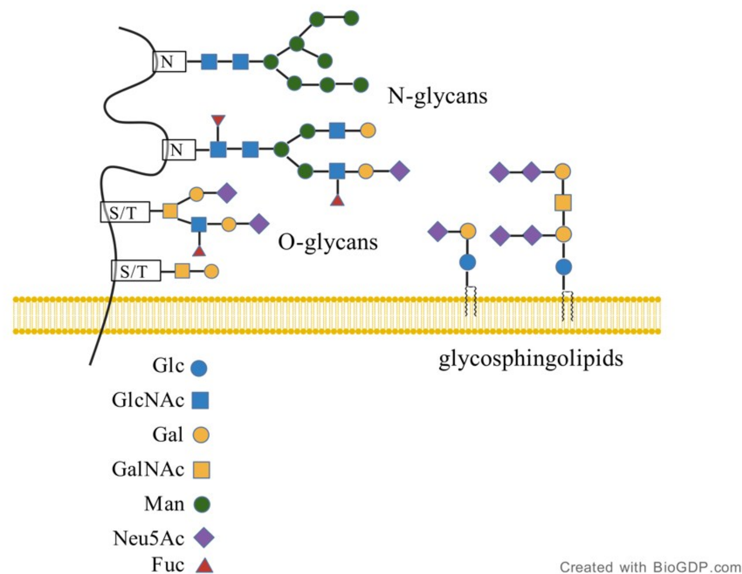

Figure 1.5: Common glycoconjugate structures in human body.

Glycoproteins typically feature two main types of glycans: N-glycans, attached to Asn residues, and O-glycans, attached to Ser or Thr residues. Glycosphingolipids (GSL) consist of one or more glycans attached to a ceramide lipid core. Adapted from Reily, C.; Stewart, T. J.; Renfrow, M. B.; Novak, J. Glycosylation in Health and Disease. *Nat. Rev. Nephrol.* **2019**, *15* (6), 346–366. (Figure was created with BioGDP.com)[18]

In recent decades studies have highlighted the critical roles of glycans in cellular function, metabolism, and recognition.[28] Glycosylation serves as a regulation mechanism in processes such as cell recognition, signal transduction, receptor-ligand interactions, and other intra- and intercellular activities. As a post-translational modification, glycosylation primarily happens in the endoplasmic reticulum (ER) and Golgi apparatus, where modified proteins and lipids are subsequently transported to the plasma membrane, contributing to cellular interactions and communication.[29,30] A

specific example of glycosylation modulating the function of a receptor was demonstrated 30 years ago by Sako et al., who found that desialylation of PSGL-1 markedly impairs its ability to bind selectins.[31] Thus, specific glycan structures serve as recognition elements in selectin-mediated processes and may be regulated by cellular processes, such as biosynthesis or processing of glycans.

1.3.2 Glycoprotein modulation

Changes in the glycosylation of glycoprotein receptors and their ligands can play a significant role in the regulation of inflammation and infection responses.[32,33] For instance, as previously discussed, sLeX is a vital epitope on PSGL-1, facilitating high-affinity binding with selectins during leukocyte rolling. In contrast, the desialylation of sLeX (CD15s) by native NEU enzymes results in the exposure of Lewis X (LeX, CD15), which is essential for myeloid differentiation and serves as a key marker for mature myeloid cells.[34] However, LeX exhibits markedly reduced binding affinity to selectins compared to sLeX, significantly impairing the efficiency of PSGL-1 mediated adhesion.[31] Previous work in our group has investigated human NEU enzymes in LFA-1 mediated adhesion. In those studies, exogenous NEU enzymes were used to alter cellular glycosylation which resulted in changes to LFA-1 receptor conformation and adhesion.[35]

1.3.3 Glycolipid modulation

Glycosphingolipids, a subtype of glycolipids containing a ceramide core, can form specialized domains in the membrane sometimes referred to as “lipid rafts”. These domains are proposed to alter the interactions with components in the extracellular

matrix to regulate integrin function.[36] For example, a study has shown that disrupting the integrity of membrane rafts by decreasing cholesterol levels impairs the functional interactions of LFA-1.[37] This finding suggests that membrane rafts provide a critical platform to support LFA-1 adhesion and rapidly modulate its function during inflammatory responses.[37] Additionally, during LFA-1 activation via inside-out or outside-in signaling pathways, membrane rafts may regulate conformational changes leading to the activated state.[38] Glycolipid composition of the membrane may also regulate the endocytosis and transport of integrin receptors.[39,40] Collectively, these findings highlight how membrane glycans may contribute to modulation of LFA-1 function within the inflammatory cascade.

1.3.4 Glycosylation modulation: the role of sialic acid

Sialic acid (*N*-acetylneuraminic acid, Neu5Ac, the structure as shown in **Figure 1.6**), is a monosaccharide commonly added as the terminal residue during glycan modification. This terminal modification serves as a critical regulation mechanism, as alterations in sialic acid residues dynamically respond to cellular activities.[41] As discussed above, sialic acids may form essential components of recognition epitopes for glycan-binding proteins.[31,42] Glycolipids containing sialic acids are known as gangliosides, and are terminated by one or more sialic acid residues.[35] The addition and removal of sialic acid from glycoconjugates requires two different enzyme families, neuraminidases (NEU) and sialyl-transferases (ST). In mammalian glycosylation, core glycan structures are generated in the ER and Golgi. Sialic acids are then added by STs that cap glycans via α2,3, α2,6, or α2,8 linkages (as shown in **Figure 1.6**) in the Golgi

apparatus, after which the mature glycoproteins and glycolipids are secreted to the plasma membrane.[43] On the other hand, NEU enzymes mediate the removal of sialic acids, sometimes referred to as desialylation. The resulting asialo-proteins or lipids may then be endocytosed from the plasma membrane to the lysosome for degradation[44] or recycled back to the Golgi for re-sialylation and subsequent re-exportation to the membrane.[45] Together ST and NEU enzymes maintain the complement of sialo- and asialo-glycoconjugates in the cell,[44,46] and their activity may serve to regulate adhesion receptors and ligands involved in inflammation. Less work has been focused on understanding human NEU activity in inflammation, however the importance of sialic acid in immunoreceptor recognition and inflammatory processes may suggest they could serve critical regulatory roles.[47]

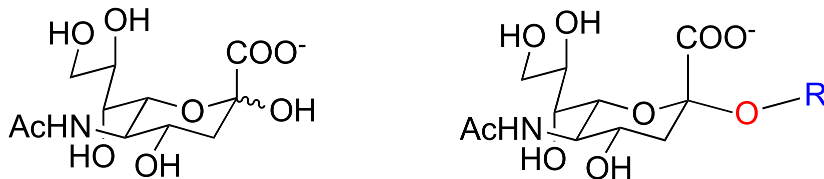

Figure 1.6: The structure of sialic acid

The structure of sialic acid is shown on the left. On the Right, the linkage between the red oxygen and the blue R group (representing the rest of residues) is an α2,3, α2,6, or α2,8 linkage, depending on the type of STs.

1.4 Human neuraminidases as regulators in the inflammatory cascade

1.4.1 The family of human neuraminidase enzymes

Neuraminidases are enzymes which remove terminal sialic acids by cleaving the glycosidic linkage of glycans. These enzymes are almost exclusively exo-α-sialidases (EC 3.2.1.18) that may cleave α2,3, α2,6, or α2,8 linkages, and thus play an important role in regulating glycan structure. There are four isoenzymes of neuraminidase in humans: NEU1, NEU2, NEU3, and NEU4; each of these enzymes have distinct subcellular localizations and substrate preferences.[44] For example, NEU1 is predominantly localized in lysosomes and partly on the plasma membrane, where it preferentially acts on glycoproteins.[48] The NEU3 isoenzyme, by contrast, is associated with plasma, endosomal, and lysosomal membranes, and exhibits a strong preference for glycolipids such as GM3.[48] NEU2 is localized to the cytosol, while NEU4 is distributed across multiple cellular sites.[48] NEU4 can desialylate glycoproteins and glycolipids.[47] These enzymes are proposed to participate in biological processes including malignancy, oncogenesis, apoptosis, and the inflammatory cascade.[49,50,51]

In the context of inflammation, the modulation of glycosylation by NEU may influence key events such as leukocyte adhesion. The leukocyte adhesion cascade, mediated by receptors including selectins, integrins, and their ligands, includes glycoproteins with terminal sialic acid residues. As sialic acid can be a critical component of recognition epitopes for these systems or affect protein conformation, native NEU activity could act as a regulator of these interactions.[34,52]

1.4.2 The roles of NEU isoenzymes in inflammation

Neuraminidase enzymes, and sialic acid, have long been recognized to contribute to the function of immune cells and immunoreceptor systems. Over 40 years ago it was discovered that treatment of immune cells with exogenous neuraminidase enzymes enhanced lymphocyte antigen presentation.[53] Native sialidases are known in lymphocytes, and have long been proposed to be involved in cellular activation.[52,54] Neutrophil sialidase activity has been found to convert sLeX antigens (CD15s) to their asialo form (CD15).[34] Many of these early studies did not investigate the identity of NEU isoenzymes involved in these processes. Considering that cells may express four isoenzymes with overlapping activity, it is essential to understand the precise roles of these isoenzymes in inflammation.

Our group has previously investigated the roles of NEU isoenzymes in regulating leukocyte adhesion receptors. Howlader et al. found that exogenous NEU3 enzyme activity could negatively regulate LFA-1 adhesion, conformation, and lateral mobility.[35] NEU3 was found to enhance the lateral mobility of LFA-1 in the plasma membrane while inhibiting its adhesion function, suggesting a negative regulation role in leukocyte LFA-1 adhesion.[35] Howlader et al. observed that NEU3 activity could modify both glycolipid content of the cells and alter the sialylation of the LFA-1 glycoprotein.[35] Using a murine model of leukocyte recruitment, Howlader et al. found that NEU isoenzymes had disparate roles in inflammation.[55] Using a lipopolysaccharide (LPS)-induced air pouch model in knock-out (KO) and wild-type (WT) mice, they demonstrated that NEU1 and NEU3 positively regulate leukocyte infiltration. The NEU1 and NEU3 KO mice exhibited lower infiltration compared to WT mice.[55]

Conversely, NEU4 KO mice displayed enhanced leukocyte trafficking, indicating an opposing regulation role.[55] Additional in vitro studies have found that one mechanism that may account for the role of NEU in leukocyte recruitment is the regulation of β1 integrins involved in transmigration.[56]

These findings suggest that NEU may participate in the regulation of components of the inflammatory cascade, and that there are isoenzyme-specific aspects of NEU that remain to be elucidated. An important research tool in these studies has been knockdown of enzyme expression using shRNA and the development of selective small molecule inhibitors for NEU isoenzymes. By targeting these enzymes, we hope to uncover their precise contributions to LFA-1 function and their potential as therapeutic targets in modulating inflammation and immune responses. Moreover, except for their roles in leukocyte inflammation responses, hNEU isoenzymes have also been associated with disease progression and identified in human blood serum.[57]

1.5 Human neuraminidase in blood

Neuraminidases play a crucial role in a variety of biological processes, including cellular signaling, immune responses, and the modulation of glycosylation.[44,47,58] As previously discussed, there are four isoenzymes in the human NEU family, and each vary in tissue distribution, substrate specificity, and functional roles.[44] Among these isoenzymes, NEU1 and NEU3 are the most studied. NEU1 is primarily localized in the lysosomes, where it degrades improperly folded glycoproteins, or other glycoconjugates in response to cellular signals.[44] NEU3 is predominantly found on the membranes, so that it is involved in regulating receptor-ligand interactions and cell surface

glycosylation.[59] Although serum NEU activity has been known for some time; recently, the NEU1 and NEU3 isoenzymes have been identified in human serum.[57]

Serum is the soluble component of human blood without anticoagulants. It contains various proteins such as albumin and antibodies, as well as enzymes, including neuraminidases, which are secreted by different cell types. The activity of serum neuraminidase has been reported to correlate with individual conditions, like immune regulation, metabolism, and disease progression.[60] Recent studies have demonstrated that NEU1 and NEU3 are the predominant isoenzymes in blood serum.[57] Notably, higher serum levels of NEU3 and lower levels of NEU1 have been observed in cancer patients compared with healthy individuals, such as prostate cancer, colon cancer, and other cancers.[57] Additionally, some studies have shown serum NEU activity is seasonally sensitive, especially in winter, likely due to increased prevalence of influenza and colds.[61] It suggested the activities of serum NEU can be influenced by environment factors in response to the bodily needs to regulate metabolism.[61]

These findings indicate the activity of serum NEU is dominated by NEU1 and NEU3, and is affected by disease progression, particularly cancer.[57] Therefore, serum NEU could serve as a potential biomarker for cancer therapy, and measurement of serum NEU activity provides a diagnostic option for early cancer.[49,57,59] Targeting these enzymes with selective inhibitors presents a promising strategy for therapeutic intervention.

1.6 Inhibitors of NEU isoenzymes

The findings discussed above provide insight into the distinct roles of NEU isoenzymes in leukocyte adhesion and serum. An improved understanding of the mechanisms that NEU may regulate will help determine the therapeutic potential of targeting specific isoenzymes. To advance this approach, our group has designed multiple selective inhibitors for hNEU isoenzymes: CG33300 for hNEU1, CR96300 for hNEU3, and CY16600 for hNEU4.[62–65] Inhibitors have been tested using recombinantly expressed and purified NEU isoenzymes. These compounds have previously been used for in vitro and in vivo studies of NEU function.[66–69] Each inhibitor exhibits prominent selectivity and potency for its respective isoenzyme. CG33300 demonstrated a K_i value of 53 ± 5 nM, with a 330-fold selectivity over other isoenzymes.[64] CR96300 presented a K_i value of 120 ± 10 nM, exhibiting more than 500-fold selectivity.[65] Similarly, CY16600 achieved a K_i value of 30 ± 19 nM with 500-fold selectivity, highlighting its specificity.[70]

In addition to these selective inhibitors, there are several commercially available inhibitors for human NEU. For instance, 2-deoxy-2,3-didehydro-*N*-acetyl neuraminic acid (DANA) effectively inhibits all hNEU isoenzymes. Similarly, zanamivir (ZAN) and oseltamivir carboxylate (OC) are widely used as anti-influenza drugs targeting viral NEU, and may act as non-selective inhibitors for hNEU. ZAN inhibits NEU3 and NEU4, while OC exhibits inhibition effects on all isoenzyme types.[71] These inhibitors represent promising tools for investigating the roles of human NEU isoenzymes in inflammation

and leukocyte adhesion. Furthermore, they may be essential to testing NEU as potential therapeutic agents for disease.

1.7 Hypothesis and objectives

Glycosylation plays a crucial role in inflammatory processes, including LFA-1 adhesion during the inflammatory response.[72] LFA-1 itself is a glycoprotein complex with approximately 20% of its mass composed of glycans, and that is heavily sialylated.[73,74] LFA-1 is well known to be regulated by conformational changes, and integrins have been found to exhibit conformational changes upon changes to glycosylation.[75] However, the precise role of human NEU enzymes in the regulation of LFA-1 is not well understood.

The central hypothesis of this thesis is that glycan structure, and in particular sialic acid content, of LFA-1 regulates its affinity and binding to ICAM-1. This effect may be either through direct effects on the glycan structure of LFA-1 or else by changes in other glycans in the membrane (e.g. other receptors or glycolipids.) We further propose that individual NEU isoenzymes contribute differently to these regulatory mechanisms. To test this hypothesis, we employed highly selective pharmacological inhibitors of human NEU isoenzymes. Inhibitors tested include: CG33300 (a NEU1 inhibitor), CR96300 (a NEU3 inhibitor), and CY16600 (a NEU4 inhibitor). Using these tools, we aim to demonstrate the specific roles of NEU1, NEU3, and NEU4 in the regulation of LFA-1 adhesion and explore their therapeutic potential in modulating inflammatory responses.

In Chapter II, we describe investigation of NEU isoenzymes as regulators of the interaction between LFA-1 and ICAM-1 in T cell and peripheral blood mononuclear cell (PBMC) models. Using these two models, we confirm that the effects of NEU are not limited to model T cells (Jurkat), but are also observed in PBMC models. To achieve the study objectives, we employed fluorescent carboxylate-modified beads and flow cytometry-based analysis.

In Chapter III, we hypothesized that different types of NEU isoenzymes have different activity levels in blood serum. While most studies about serum NEU activity have not specified the isoenzyme types, currently there is not a simple method for directly detecting the activity of individual isoenzyme in blood serum. To address this shortcoming, we sought to develop a method that can detect the activity of individual isoenzymes in serum using selective inhibitors.

In Chapter IV, we present conclusions from these studies and propose potential future directions to advance the understanding of hNEU in inflammatory pathways and their therapeutic implications.

Chapter II

Human neuraminidase effects on LFA-1–ICAM-1 adhesion[1]

[1] This work was supported by the Natural Sciences and Engineering Research Council of Canada (NSERC, RGPIN 2020-04371) and the National Institutes of Health (R01-HL153264); A version of this chapter is currently in review. Inhibitor compounds were prepared by Drs. Elisa Garcia Carvajal and Mostafa Radwan. Prof. J Acker and Sanaz Hemmatibardehshahi (University of Alberta) assisted with donor sample collection. DiCBM40-Alexa Fluor647 were provided by Prof. L. Mahal (U Alberta).

2.1 Introduction

Recent studies have provided substantial evidence that inflammation serves as a trigger for various diseases, including obesity, bowel disease, atherosclerosis, and cancer.[5,6,76] However, this does not imply all instances of inflammation lead to acute diseases. It is primarily uncontrolled or prolonged inflammation that contributes to pathological outcomes.[6] When inflammation occurs, it initiates inflammatory responses, including a critical process known as leukocyte recruitment, or leukocyte adhesion cascade. This process consists of four main steps: capture, rolling, firm adhesion, and extravasation.

The capture and slow rolling phases are mediated by selectins and their respective ligands, such as L-selectins, E-selectins, P-selectins, and PSGL-1 (P-selectin glycoprotein ligand 1).[13] During leukocyte rolling, chemokines activate integrins that facilitate firm adhesion and arrest leukocytes instantaneously, followed by extravasation.[20] A crucial integrin involved in firm adhesion is LFA-1 (lymphocyte function-associated antigen 1) and its ligand ICAM-1 (intercellular adhesion molecule 1). LFA-1 is a glycoprotein expressed on leukocytes, and modulating its glycosylation is an efficient mechanism for regulating inflammatory responses.[32] Glycosylation can be modulated either directly on glycoprotein receptors or via glycolipids in membrane microdomains.[35]

In the glycosylation process, sialic acid (N-acetylneuraminic acid, Neu5Ac) often serves as the terminal residue to modify proteins or lipids, and its modulation is implicated in various biological regulation pathways. The addition and removal of sialic acid residues are catalyzed by two opposing types of enzymes: sialyltransferases, which

transfer sialic acids, and neuraminidases remove them. Humans express four isoenzymes of neuraminidase enzymes (hNEU): these are: NEU1, NEU2, NEU3 and NEU4.[44] These enzymes have a variety of proposed roles including in malignancy, oncogenesis, inflammation, and adhesion.[49,55,77] Our group has found that hNEU can affect integrin-mediated cell migration, transmigration, and static-adhesion.[35,56,68,78] In previous work we tested the role of exogenous hNEU3 enzymes to modulate LFA-1 adhesion, and we observed that the enzyme was a negative regulator.[35] In this study, we aim to present the effects of other hNEU isoenzymes on LFA-1-ICAM-1 adhesion employing selective inhibitors for hNEU isoenzymes.

Currently, there are three commercial inhibitors for NEU: DANA (2-deoxy-2,3-didehydro-*N*-acetyl neuraminic acid), ZAN (zanamivir), and OC (oseltamivir carboxylate). DANA is the most widely used inhibitor for NEU, while ZAN and OC are primarily employed as anti-influenza drugs for viral NEU.[79] However, these inhibitors exhibit non-selective inhibition of hNEU isoenzymes, which limits the ability to study the roles of specific hNEU isoenzymes.[71] Thus, to address this limitation, our group has developed highly selective inhibitors: CG33300 for hNEU1, CR96300 for hNEU3, and CY16600 for hNEU4. These highly selective inhibitors provide an effective platform for directly investigating the roles of native hNEU isoenzymes in vitro cell culture models. In this study, we examined the effects of hNEU on LFA-1-ICAM-1 adhesion in both a T cell model and peripheral blood mononuclear cell (PBMC) model. Our findings demonstrated that hNEU3 negatively regulates the static adhesion of LFA-1-ICAM-1, while hNEU1 and hNEU4 act as negative regulators of both activated and static adhesion. These results suggest that hNEU play inhibitory roles in LFA-1

mediated adhesion during leukocyte recruitment, indicating their potential as therapeutic targets for modulating inflammatory responses.

2.2 Results & Discussion

2.2.1 Measurement of LFA-1–ICAM-1 adhesion by cytometry

In order to study the effects of hNEU on LFA-1–mediated adhesion, we employed an in vitro cytometry assay.[35,80] Fluorescent carboxylate microspheres were labelled with recombinant ICAM-1 and binding to cells was determined by flow cytometry. In order to activate LFA-1 adhesion, we tested treatment of cells with phorbol 12-myristate 13-acetate (PMA) or $MgCl_2$.[80,81] PMA is a protein kinase C activator, which may stimulate multiple pathways in immune cells. Divalent metal ions are thought to activate LFA-1 via binding to the metal ion dependent adhesion sites (MIDAS) in the I domain of the CD18 subunit.[26,81] As expected, both conditions activated LFA-1–ICAM-1 adhesion (**Figure 2.1**). We found that PMA treatment could enhance adhesion, however this effect was also sensitive to the time of incubation and required the use of DMSO (**Figure 2.1a**). A comparison of $MgCl_2$ and PMA treatment found that DMSO alone had a significant effect on adhesion while both activation conditions gave similar increases in adhesion relative to controls (**Figure 2.1b**). Based on these results, we proceeded to investigate effects on LFA-1–ICAM-1 adhesion in vitro with $MgCl_2$ activation.

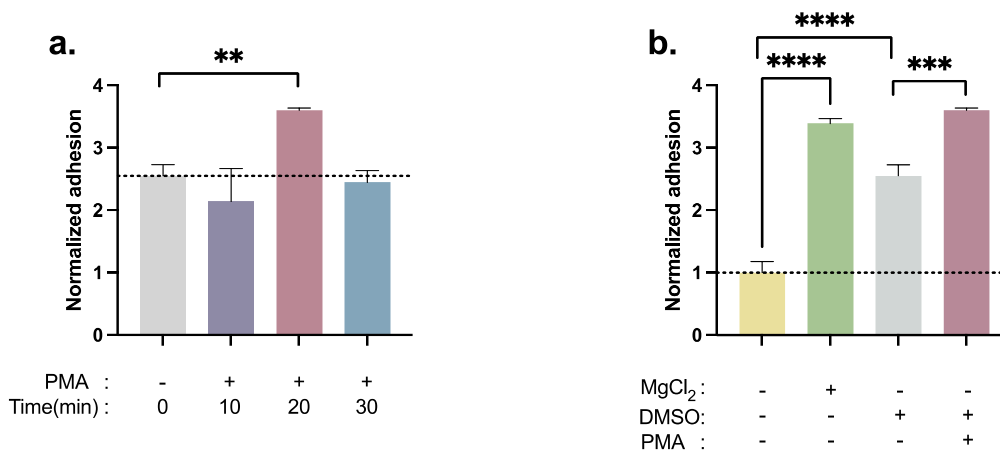

Figure 2.1: Activation conditions for LFA-1–ICAM-1 adhesion.

(**a**) Jurkat cells were treated with PMA (200 ng mL^{-1}, in 0.04% DMSO at 37 °C) for 10, 20, or 30 min followed by incubation with ICAM-1 beads for another 30 min. (**b**) Jurkat cells were treated with $MgCl_2$ (2 mM at 25 °C for 15 min) or PMA (200 ng mL^{-1}, in 0.04% DMSO at 37 °C) for 20 min; followed by incubation with ICAM-1 beads for another 30 min at 37 °C. Adhesion of beads was determined by flow cytometry, and is represented as the mean of 3 replicates for each treatment, error bars shown are for the standard deviation. Data were compared by one-way ANOVA and Student's t-test (**, $p \leq 0.005$; ***, $p \leq 0.0005$; ****, $p < 0.0001$).

2.2.2 LFA-1–ICAM-1 adhesion is suppressed by native hNEU activity

With an adhesion assay in place, we proceeded to test pharmacological agents that could reveal a role for natively expressed hNEU in regulating LFA-1 adhesion. We have previously reported the design and synthesis of isoenzyme-selective hNEU inhibitors.[64,65,70,82] For the current study, we selected representative inhibitors selective for NEU1, NEU3, and NEU4 isoenzymes. Compounds are referred to here based on

compound numbers, selectivity was determined based on relative IC_{50} of the target and the next-best isoenzyme, and compound structures are provided in **Figure 2.2**. Among these we included: CG33300, a NEU1 inhibitor (K_i = 53 nM, 330-fold selective)[64]; CR96300, a NEU3 inhibitor (K_i = 120 nM, >500-fold selective)[65]; and CY16600, a NEU4 inhibitor (K_i = 30 nM, 500-fold selective).[70] We also included compounds that are known or proposed to inhibit hNEU, these were: 2-deoxy-2,3-didehydro-*N*-acetyl neuraminic acid (DANA; a pan-selective inhibitor with IC_{50} of 2-25 μM for hNEU); zanamivir (ZAN; IC_{50} of 1-26 μM for NEU2, NEU3, and NEU4); and oseltamivir carboxylate (OC; IC_{50} > 500 μM for NEU1-4).[83] This panel of compounds presents a range of activity and selectivity for native hNEU isoenzymes.

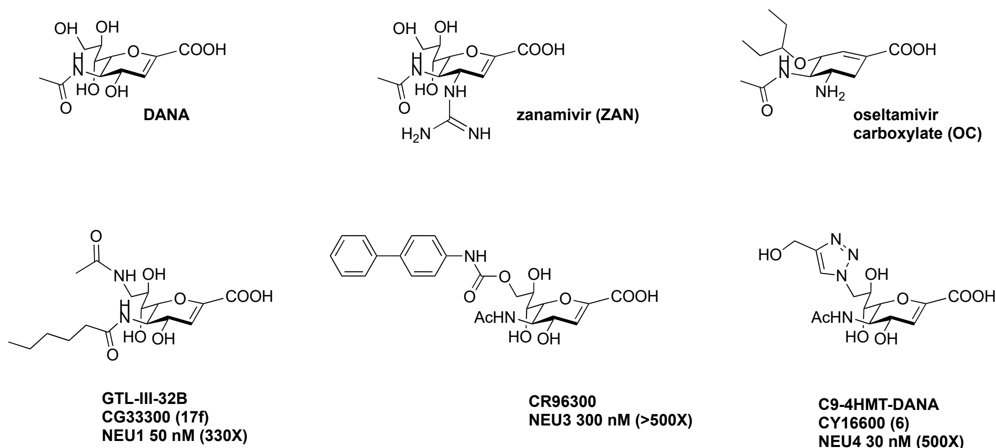

Figure 2.2: Structures of inhibitor compounds used in this study.

Inhibitors are labelled with their compound numbers, as well as the compound numbers used in the original report.

We proceeded to test the effects of these inhibitors in the LFA-1–ICAM-1 adhesion assay (**Figure 2.3**). We treated a cultured T cell model (Jurkat) with inhibitors (100 μM), cells were either untreated or activated by treatment with $MgCl_2$ as indicated.

Adhesion of cells was compared to controls for a *resting* ($MgCl_2$ / inhibitor; -/- vs. -/+) or *activated* ($MgCl_2$ / inhibitor; +/- vs. +/+) state in the absence and presence of each inhibitor (for a summary see **Table 2.1**). We first tested isoenzyme-selective inhibitors for hNEU. Selective inhibitors of NEU1 (CG33300) and NEU4 (CY16600) showed significant enhancement of adhesion in both resting and activated cells (**Figure 2.3a**, **2.3c**). However, an inhibitor of NEU3 (CR96300) only enhanced adhesion of resting cells (**Figure 2.3b**). These results agree with experiments that found exogenous hNEU3 enzyme activity inhibited LFA-1 adhesion.[35] We next tested the effect of commercially available inhibitors with broader specificity for hNEU enzymes. The viral NEU inhibitor, ZAN, activated adhesion in both resting and activated cells (**Figure 2.3d**). In contrast, DANA and OC had no significant effects on adhesion for resting or activated cells (**Figure 2.3e**, **2.3f**).

These results suggest that both potency and selectivity of hNEU inhibitors play a role in their affects on adhesion. Selective inhibitors of NEU1, NEU3, and NEU4 all enhanced LFA-1 adhesion resting cells (CG333000, CR96300, and CY16600). ZAN, which has mixed activity for NEU3 and NEU4 had a similar effect to NEU3 and NEU4 inhibitors; however, the pan-selective inhibitor DANA had no effect on resting cells. Selective compounds also provided an enhancement of adhesion in activated cells (**Figure 2.3a-d**). Compounds with lower potency and selectivity (DANA, OC) had no detectable effect on activated cells. Since this experiment was designed to screen multiple inhibitors, we did not test a range of concentrations. We next tested the dose-dependent effects of the most active compounds.

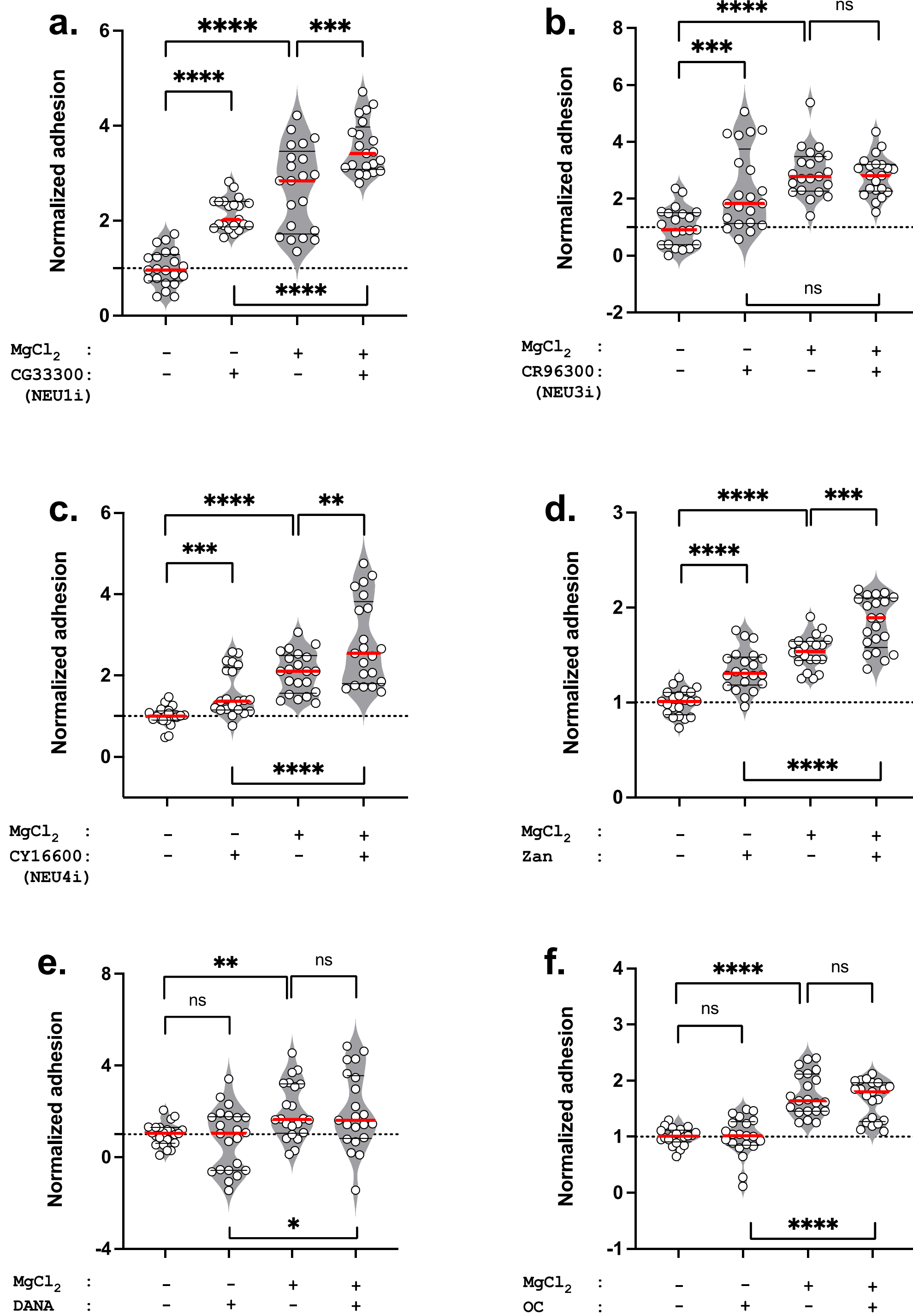

Figure 2.3: The effect of NEU inhibitor treatment on LFA-1–ICAM-1 adhesion.

Cells were treated with inhibitors as indicated (100 µM at 37 °C for 3 hrs), followed by activation where indicated (2 mM Mg^{2+}, 25 °C for 15 min). Inhibitors were (**a**) CG33300 (NEU1i), (**b**) CG96300 (NEU3i), (**c**) CY16600 (NEU4i), (**d**) Zanamivir, (**e**) DANA, and (**f**) OC. Adhesion was then determined by incubation with ICAM-1 beads (30 min at 37 °C) and analysis by cytometry (21 replicates for each concentration over 3 experiments.) Results were normalized and compared to untreated controls (dashed line) by one-way ANOVA and Student's t-test (*, $p \leq 0.05$; **, $p \leq 0.005$; ***, $p \leq 0.0005$; ****, $p \leq 0.0001$). The mean of each condition is shown by a red line, and error bars shown are standard deviation.

Inhibitor	**CG33300**	**CR96300**	**CY16600**	**Zan**	**DANA**	**OC**
Target †	**NEU1**	**NEU3**	**NEU4**	**NEU2/3/4**	**NEU1/2/3/4**	**na**
Condition						
Resting	****	***	***	****	ns	ns
Activated	***	ns	**	***	ns	ns

Table 2.1: Summary of the effects of hNEU inhibitors on LFA-1–ICAM-1 adhesion.

†, Enzymatic target of each inhibitor is given based on in vitro inhibition data.[83] *p* values from a Student's t test are indicated from the data shown in Figure 2 for *resting* (comparing control [-/-] to inhibitor alone [-/+]) and *activated* (comparing activated [+/-] to activated with inhibitor [+/+]) conditions.

We designed a modified experiment to compare the selective inhibitors CG33300, CR96300, and CY16600 over a range of concentrations (10 – 500 µM, **Figure 2.4**). For this study, cells were untreated (resting) and adhesion was measured using the same protocol as above after treatment with inhibitors at the indicated concentrations. The hNEU1 inhibitor, CG33300 had its maximal effect at 50 – 100 µM,

and at higher concentrations (> 200 μM) appeared to return to the untreated control (**Figure 2.4a**). The hNEU3 inhibitor CR96300 reached a maximal increase in adhesion at 100 μM, which was sustained at higher concentrations up to 500 μM (**Figure 2.4b**). Compound CY16600, a hNEU4 inhibitor, followed a similar pattern to the hNEU1 inhibitor with a maximum at 50 – 100 μM (**Figure 2.4c**). Based on these results, we concluded that native NEU1, NEU3, and NEU4 activity negatively regulates LFA-1 adhesion and therefore competitive inhibitors enhance LFA-1 adhesion. The effect is saturable in the case of NEU3 inhibitors; however, NEU1 and NEU4 inhibitors only have an effect over a narrow concentration range. Or as the concentration of inhibitors increases, most NEU1 and NEU4 become occupied, leading to a maximum enhancement of adhesion. Their target glycoproteins become hyersialylated, potentially triggering endocytosis or other regulation mechanisms to restore normal sialylation levels, and eventually bring adhesion back to baseline. However, NEU3 primarily target glycolipids, which are associated with membrane microdomains and regulate LFA-1 function indirectly as showed in previous Howlader et al. study.[35] Unlike NEU1 and NEU4, NEU3 does not act directly on adhesion receptors, resulting in the regulation mechanisms. Due to the expression level of LFA-1, NEU3-mediated adhesion reaches saturation. Considering that inhibitors of hNEU had a significant effect on LFA-1 adhesion, we proceeded to test if these compounds had any effect on cellular glycosylation of cells.

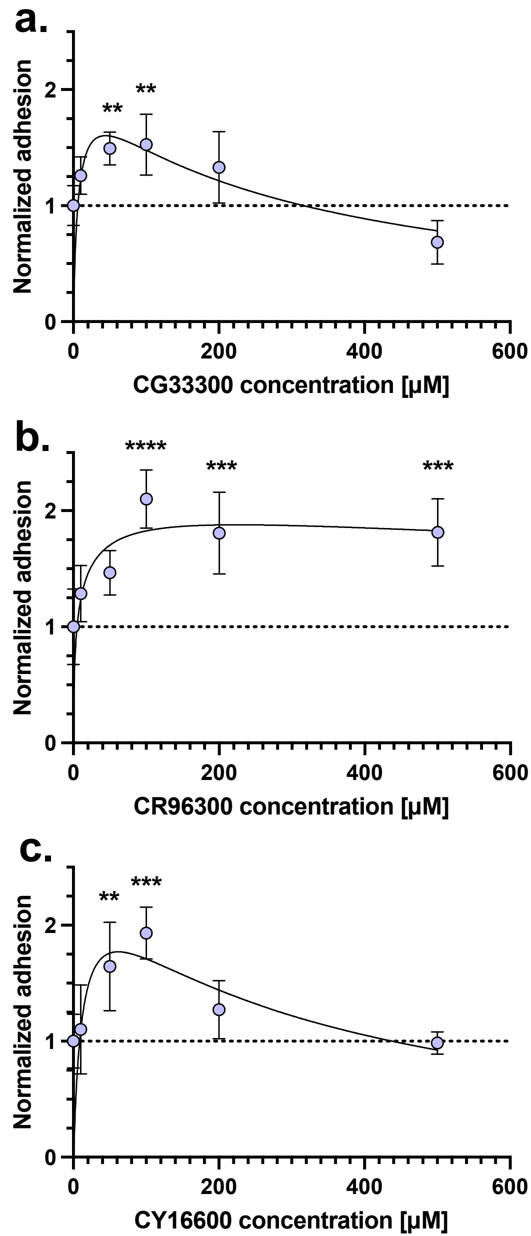

Figure 2.4: Dose-dependent effects of hNEU inhibitors on LFA-1–ICAM-1 adhesion.

Cells were treated with inhibitors at the indicated concentrations (37 °C for 3 hrs. Inhibitors were (**a**) CG33300 (NEU1i), (**b**) CG96300 (NEU3i), or (**c**) CY16600 (NEU4i). Adhesion was then determined by incubation with ICAM-1 beads (30 min at 37 °C) and analysis by cytometry (5 replicates for each concentration.) Results were

normalized and compared to untreated controls (dashed line) by one-way ANOVA (**, $p \leq 0.005$; ***, $p \leq 0.0005$; ****, $p < 0.0001$). Error bars are shown as standard deviation, and the data were fit to a Lognormal distribution to guide the eye.

2.2.3 Cellular glycosylation is altered by treatment with inhibitors of human NEU

To analyze the effect of human neuraminidase inhibitors on cellular glycosylation, we used lectin staining analyzed by cytometry. Cells were stained with fluorophore-conjugated lectins di CBM40 (diCBM40-Alexa Fluor647; which binds α2-3 and α2-6 linked sialic acids)[84] and the peanut agglutinin (PNA-Alexa Fluor647; which binds galactose).[85] As a positive control we treated cells with NanI, a neuraminidase from *Clostridium perfringens* which can cleave terminal sialic acid residues in α2-3, α2-6, and α2-8 linkages from glycoproteins and glycolipids.[86] As expected, NanI treatment of cells significantly reduced diCBM40 staining, indicating that sialic acid residues were removed. This same treatment gave a substantial increase in PNA staining, consistent with sialidase activity revealing Gal (galactose) binding sites (**Figure 2.5**). All NEU inhibitors tested resulted in increased diCBM40 staining, with the NEU4 inhibitor showing an almost 4-fold increase. After PNA treatment, all inhibitors except OC showed a small reduction in staining. However, Cosmc may affect PNA staining. As reported, the core1 β3-galactosyltranferase-specific molecular chaperone (Cosmc) is essential for the proper function of core1 β3-galactosyltranferase, which transfers a galactose to the Tn antigen (GalNAcα1-Ser/Thr) to form the T antigen (core1 O-glycan Galβ1-3GalNAcα1-Ser/Thr).[87] In Jurkat cells, mutations or dysfunction in Cosmc lead to an accumulation of Tn antigens on the cell surface.

Consequently, when Jurkat cells are stained with PNA, the signals could be reduced due to the abnormal or misfolded core1 β3-galactosyltranferase.

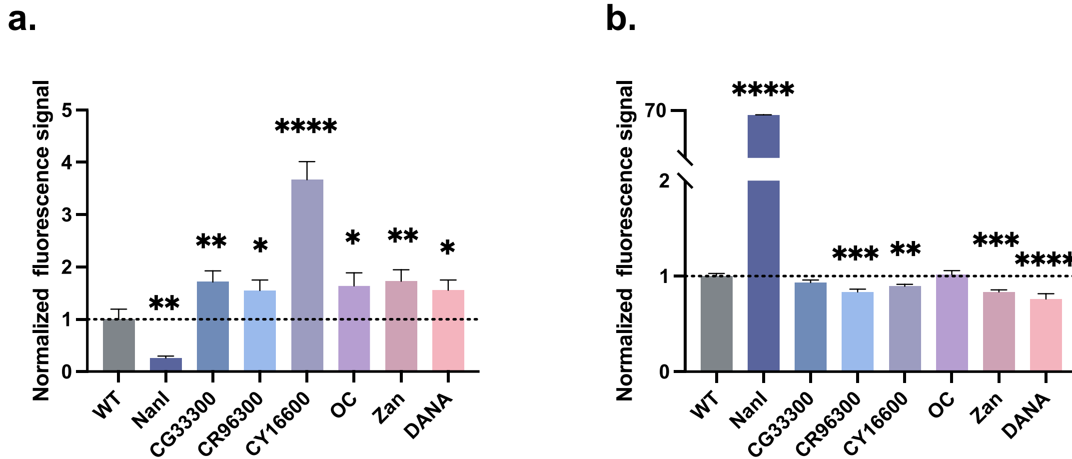

Figure 2.5: Effect of NEU inhibitor treatment on cell glycosylation.

Jurkat cells were treated as indicated (16.8U of NanI (2 hrs) or inhibitors at 100 µM for 3 hrs at 37 °C) and then stained with (**a**) diCBM40-Alexa Fluor647 or (**b**) PNA-Alexa Fluor647 (30 min on ice). Staining was determined by flow cytometry. Results were normalized and compared to untreated controls (dashed line) using one-way ANOVA and Student's t-test (*, $p \leq 0.05$; **, $p \leq 0.005$; ***, $p \leq 0.0005$; ****, $p < 0.0001$). The mean of three replicates is shown for each point, and error bars are standard deviation.

The lectin staining results are consistent with native hNEU activity being involved in turnover of membrane glycans, and support that the activity of the inhibitors is due to inhibition of this turnover.[88–90] Our staining protocol used intact cells and lectin conjugate staining (30 min on ice), which should primarily detect extracellular glycans. In the absence of hNEU activity, both staining conditions should have shown little or no change. The large difference in diCBM40 staining after NEU4 inhibitor treatment

may indicate that this enzyme is substantially more active than the other isoenzymes in this condition. Furthermore, the adhesion data indicated that OC had no effect on LFA-1 adhesion while this compound did alter diCBM40 staining. This may indicate that there are specific sialoside targets of hNEU which affect LFA-1 adhesion, rather than this being a general effect. Finally, we note that the inhibitors may be cell permeable and could block lysosomal enzyme activity; although many of these compounds are expected to have relatively low log P values.[63,65]

2.2.4 Interaction of NHE1 inhibitors with LFA-1–ICAM-1 adhesion and hNEU

After establishing that hNEU inhibitors could modulate LFA-1 adhesion, we attempted to gain more mechanistic insight into this effect by testing additional conditions in our adhesion assay (**Figure 2.6**). We first confirmed that the assay is detecting LFA-1–mediated adhesion by confirming that a blocking antibody, TS1/22, inhibited adhesion after activation with $MgCl_2$.[26] To lend further support that we are detecting LFA-1 adhesion, we tested the small molecule inhibitor BIRT377, an allosteric inhibitor that blocks the high-affinity conformation of LFA-1.[91,92] Due to the hydrophobic nature of this compound, it was dissolved with a low concentration of DMSO.[92] Comparing BIRT377 treatment with vehicle controls confirmed that the compound blocked LFA-1 adhesion after cell activation with $MgCl_2$. These experiments validate the adhesion assay is specific for LFA-1–mediated adhesion.

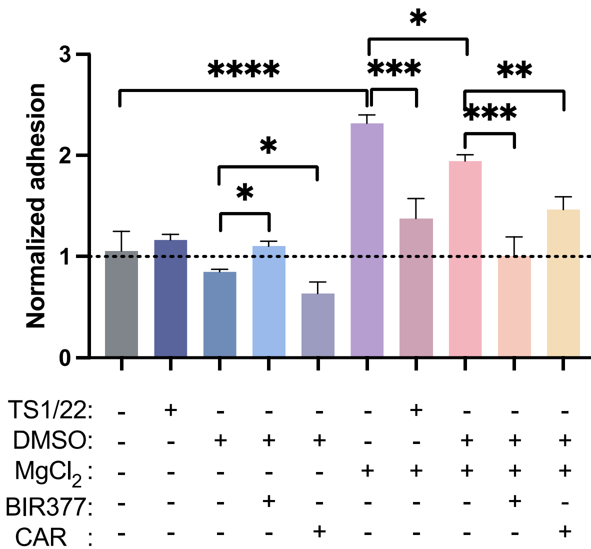

Figure 2.6: The effect of LFA-1 and NHE-1 inhibitors on adhesion.

Jurkat cell adhesion to ICAM-1 coated beads was determined by flow cytometry after cells were treated with the indicated treatments: TS1/22 (3.6 μg mL^{-1}), BIRT377 (10 μM), CAR (200 nM), and DMSO (0.01%). Where indicated, cells were first activated by incubation with Mg^{2+} (2 mM, 30 min at 25 °C). Adhesion was then determined by incubation with ICAM-1 beads (30 min at 37 °C) and analysis by cytometry (3 replicates for each condition.) Results were normalized and compared to untreated controls (dashed line) by one-way ANOVA (*, $p \leq 0.05$; **, $p \leq 0.005$; ***, $p \leq 0.0005$; ****, $p < 0.0001$). Error bars are shown as standard deviation.

The pH optimum of hNEU enzymes is acidic due, which may suggest that they are primarily active in the lysosomal compartment. However, it has it has been suggested that hNEU enzymes may be active at the plasma membrane through the assistance of Na+/H+ exchangers, such as NHE1.[93,94] Small molecule inhibitors of NHE1 are available, and we decided to test the effects of cariporide (CAR) in our adhesion assay.[95] CAR was dissolved in DMSO and compared with a DMSO vehicle

control. Interestingly, CAR alone reduced LFA-1 adhesion below the resting state, and CAR treatment after MgCl2 activation was significantly reduced relative to controls. These findings are consistent with NHE1 acting to enhance hNEU activity, but do not rule out other mechanisms for NHE1 effects on LFA-1 adhesion.

We next determined that CAR only affected LFA-1 adhesion at 50 – 100 nM, while higher concentrations had little or no effect (**Figure 2.7a**). Macdonald et al. proposed that EGF signaling stimulates the NHE1 antiporter, which transiently acidifies the extracellular environment to enhance hNEU activity.[94] Hence, we hypothesized that if NHE1 is acting upstream of hNEU, CAR activity should be blocked by hNEU inhibitors. To test this, we performed a titration of the NEU1 inhibitor CG333000 in the presence of CAR (**Figure 2.7b**). Results were normalized to vehicle treatment alone (DMSO). While low concentrations of the NEU1 inhibitor (10 μM) did not block the increased adhesion from CAR, higher concentrations (≥100 μM) ablated the effects of CAR. When we analyzed the effects of these conditions on cellular glycosylation, we observed that treatment with CAR induced increased diCBM40 (~ 5-fold) and slightly decreased PNA staining (**Figure 2.8**). When CAR treatment was combined with the NEU1 inhibitor CG33300, we observed the largest increase in diCBM40 staining in this study. In contrast, PNA staining was no different from control under combined CAR and CG33300 treatment. We also observed that $MgCl_2$ and DMSO treatments were sufficient to increase diCBM40 staining and decrease PNA staining. We note that previous reports have found that CAR may activate lysosomal exocytosis,[93] and it is possible that this could account for increased diCBM40 staining. However, this mechanism would not be expected to be enhanced by NEU1 inhibition. Furthermore, if

exocytosis was the only operative mechanism, then we would also expect some change in PNA staining to accompany this treatment since lysosomes are expected to contain galectin binding sites.[96] As a result, we favor the hypothesis that NHE1 helps to maintain normal hNEU activity at the plasma membrane, and that combined inhibition of NHE1 and hNEU disrupts sialic acid turnover.

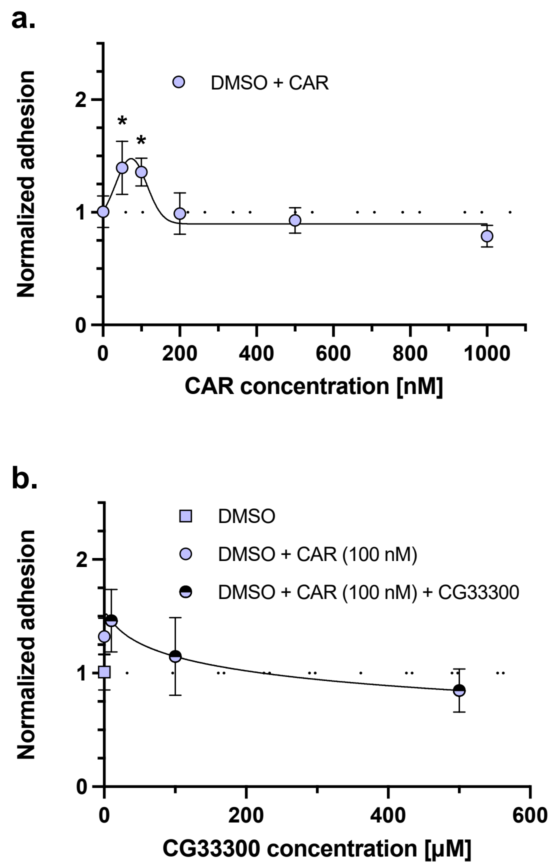

Figure 2.7: Effects of cariporide and a NEU1 inhibitor on LFA-1–ICAM-1 adhesion.

(**a**) Cells were treated with cariporide at the indicated concentrations (37 °C for 30 min in 0.001% DMSO). Adhesion was then determined by incubation with ICAM-1 beads (30 min at 37 °C) and analysis by cytometry (4 replicates for each concentration.) Results were normalized and compared to untreated control (dashed line) by one-way

ANOVA (*, $p \leq 0.05$). Error bars are shown as standard deviation, and the data were fit to a gaussian distribution to guide the eye. (**b**) Jurkat cells were treated with DMSO (0.0001%) or CG33300 as indicated, followed by cariporide (100 nM at 37 °C for 30 min in 0.0001% DMSO). Adhesion was then determined by incubation with ICAM-1 beads (30 min at 37 °C) and analysis by cytometry (4 replicates for each concentration.) Results were normalized and compared to untreated control (dashed line) by one-way ANOVA. Error bars are shown as standard deviation.

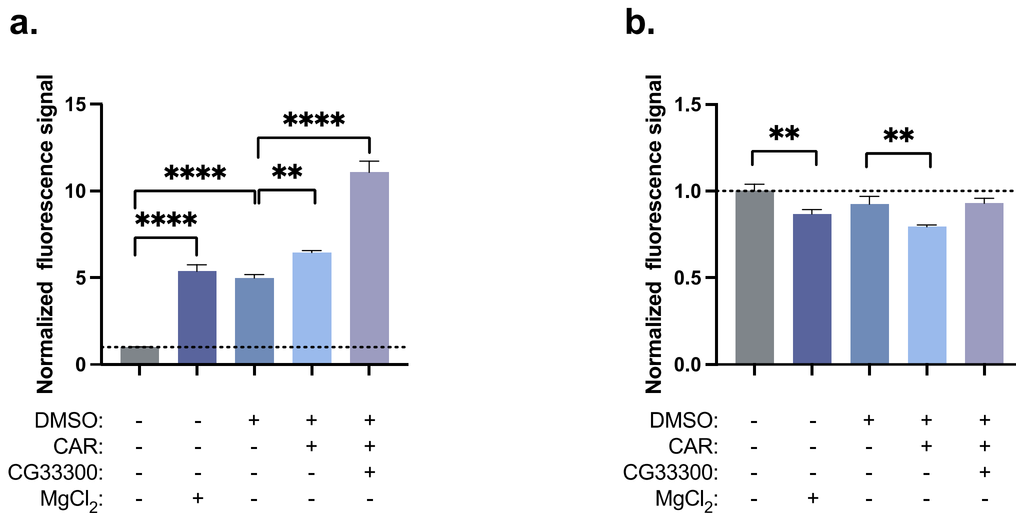

Figure 2.8: Effect of activation, cariporide, and NEU inhibitor treatment on cell glycosylation.

Jurkat cells were treated as indicated. Treatment with buffer or the NEU1 inhibitor was performed first (CG33300, 100 μM at 37 °C for 3 hrs), followed by addition of cariporide (100 nM of CAR in 0.0001% DMSO). Where indicated, cells were activated by incubation with Mg^{2+} (2 mM, 15 min at 25 °C) before incubation with ICAM-1 beads. Cells were then stained with (**a**) diCBM40-Alexa Fluor647 or (**b**) PNA-Alexa Fluor647 (30 min on ice). Staining was determined by flow cytometry. Results were

normalized and compared to untreated controls (dashed line) by one-way ANOVA (**, $p \leq 0.005$; ****, $p < 0.0001$). The mean of three replicates is shown for each point, and error bars are standard deviation.

2.2.5 LFA-1–ICAM-1 adhesion is modulated by hNEU in PBMC

Up to this point, we had performed all experiments in a cultured T cell model, and we considered that a primary leukocyte model should be used to confirm our key findings. We obtained peripheral blood mononuclear cells (PBMC) from healthy donors and tested them in our LFA-1–ICAM-1 adhesion assay (**Figure 2.9**). The cells demonstrated increased adhesion on activation with $MgCl_2$ treatment as expected. Importantly, we found that treatment of resting PBMC with NEU1 and NEU4 inhibitors (CG33300 and CY16600) increased adhesion. We confirmed that OC, which had no activity in Jurkat cells, also had no effect on the adhesion of PBMC. The lack of an effect for hNEU inhibitors on activated cells may suggest that turnover of sialoglycans in activated PBMC involves other mechanisms. Alternatively, PBMC contains multiple leukocyte cell types, unlike the Jurkat model, which consists entirely of T cells. While T cells are a major component of PBMC, other leukocyte populations may be less sensitive to hNEU inhibitor treatments in the activated state, contributing to the observed differences, although without statistical significance. Additionally, the small bio-sample group may also contribute to this variation.

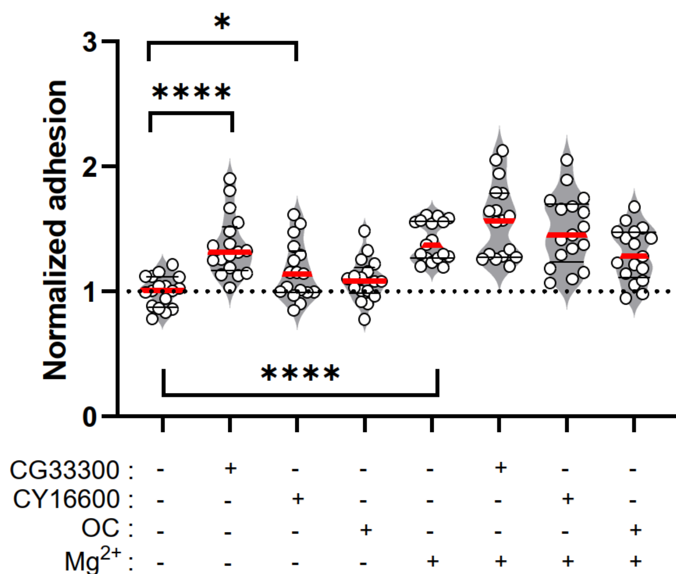

Figure 2.9: Effects of hNEU inhibition on LFA-1–ICAM-1 adhesion in PBMC. PBMC were collected from three healthy donors and used for LFA-1–ICAM-1 adhesion assays. Cells were treated with CG33300, CY16600, and OC (100 µM at 37 °C for 3 hrs). Where indicated, cells were activated by incubation with Mg^{2+} (2 mM, 15 min at 25 °C) before the adhesion assay. Adhesion was then determined by incubation with ICAM-1 beads (30 min at 37 °C) and analysis by cytometry (5-6 replicates for each condition over three experiments.) Results were normalized and compared to untreated controls (dashed line) by one-way ANOVA and Student's t-test (*, $p \leq 0.05$; ****, $p < 0.0001$). The mean of each condition is shown by a red line, and error bars shown are standard deviation.

2.3 Conclusions

This study has advanced our understanding of the role of hNEU isoenzymes in regulating leukocyte adhesion. Based on our findings, we demonstrated that hNEU can

regulate firm adhesion by modulating the glycosylation of distinct and specific targets on lymphocytes, with NHE-1 assisting in hNEU activity. However, combined inhibition of NHE-1 and hNEU was found to abolish these regulatory effects. Furthermore, the interaction between LFA-1 and ICAM-1 is mediated by conformational changes in LFA-1, such as the Mg^{2+} stimulated transition to a high-affinity state. In the T cell model, hNEU1 and hNEU4 play suppressive roles in both LFA-1 activated and static adhesion, while hNEU3 exhibits a negative effect exclusively in the resting state. Similarly, in PBMCs, we demonstrated that hNEU1 and hNEU4 maintain consistent suppressive roles in adhesion, particularly when LFA-1 is in the resting state. These findings highlight that hNEU isoenzymes are promising therapeutic targets for disease treatment, and these selective inhibitors have the potential to serve as anti-inflammatory drugs.

2.4 Materials & Methods

2.4.1 Cell culture

Jurkat cells (clone E6-1; ATCC) were maintained in RPMI 1640 medium with 10% fetal bovine serum (FBS), 1.25 μg mL^{-1} amphotericin B, 50 units mL^{-1} penicillin, and 50 μg mL^{-1} streptomycin at 37 °C with 5% CO_2 to 1 x 10^6 cells mL^{-1}. Cells were prepared for experiments by centrifugation (300 rcf, 2 min) and resuspension in HBS buffer (20 mM HEPES, 150 mM NaCl, 2 mM $CaCl_2$, pH 7.4) to 1 x 10^6 cells mL^{-1}.

Peripheral blood mononuclear cells (PBMC) were isolated from 7 – 8 mL donated whole blood of healthy donors (UAlberta HREB Protocol #Pro0016491). The sample was diluted 1:1 with sterile PBS to 15 mL. The suspension was gently overlaid

on 10 mL of Histopaque-1077 (ficoll density gradient medium, 1.077 g mL^{-1}; Sigma-Aldrich) and centrifuged (1000 rcf, 20 min). The buffy coat layer was carefully transferred to a fresh tube and washed with RPMI medium twice after centrifugation (400 rcf, 10 min). The cell pellet was resuspended in RPMI medium (5 mL) and transferred to a T-25 flask, and incubated overnight (37 °C, 5% CO_2). After 24 hrs, the cells were removed from the T-25 flask, washed, resuspended, and diluted to 1 x 10^6 cell mL^{-1} in HBS buffer.

2.4.2 Labelling of carboxylate microspheres with ICAM-1

Fluorescent carboxylate-modified microspheres (1 µm; Invitrogen) were handled in the dark. Recombinant human ICAM-1 Fc chimera protein (R&D Systems) was used as provided by the manufacturer. To label beads with ICAM-1, beads (2 µL) were activated with a fresh mixture of EDCI-HCl and NHS (1 mL, 1 mM final concentration, 4:1 stoichiometry) for 30 min. The activated beads were washed by centrifugation (19,000 rcf, 5 min; 5000 rcf, 5 min) and resuspended in MES buffer (50 mM, pH 6.0) containing 4 - 8 µg of ICAM-1 Fc chimera protein or MES buffer alone as a negative control. The bead suspension was incubated at room temperature for 2 h, followed by centrifugation (5,500 rcf, 20 min), and resuspension in ethanolamine (100 mM, pH 8.0) for 1 h to quench the activated beads. Beads were then washed with PBS containing 1% BSA (200 µL) by centrifugation (3 times, 20,000 rcf, 10 min). The beads were centrifuged a final time (5,000 rcf, 5 min) and resuspended in HBS with 0.1% BSA (500 µL) and sonicated in an ice water bath for (30 min), diluted to 1 mL in HBS buffer

and stored in the dark until used in experiments. ICAM-1 beads were used within 24 hrs of preparation.

2.4.3 LFA-1–ICAM-1 cytometry assay

Before the adhesion assay, cells were treated with inhibitors and conditions as indicated. Inhibitors of hNEU were typically used at 100 μM (CG33300, CR96300, CY16600, DANA, ZAN, OC), and incubated with cells (500 μL, 1 x 10^6 cells mL^{-1}) at 37 °C for 3 hrs. Cells were either activated with 2 mM $MgCl_2$ at 25 °C for 15 min or untreated (25 °C, 15 min), and then incubated with a suspension of ICAM-1 beads (200 μL of beads with 500 μL of cells at $1x10^6$ cells mL^{-1}) at 37 °C for 30 min. Phorbol 12-myristate 13-acetate (PMA) was used to activate cells (200 ng mL^{-1} in MQ H_2O with 0.04% DMSO; ThermoFisher). Cells were then immediately analyzed by flow cytometry with a BD Accuri C6 flow cytometer without any wash steps.

Before analysis, samples of untreated control cells were used to identify the gate (P1) for cells using forward scattering (FSC) versus sideward scattering (SSC; **Figure 2.10**). Labelled cells were counted after P1 gating using FSC vs. fluorescence (FL4) to analyze LFA-1 ICAM-1 adhesion (**Figure 2.11**). The resulting plot was divided by quadrants, with a cutoff of 10^4 for fluorescence intensity. Signals in the upper-right quadrant (**Figure 2.12**) indicated cells with ICAM-1 beads bound. The background binding of blank beads (without ICAM-1) with cells was used as the blank. Adhesion was then calculated using the following equation:

$$\text{Normalized adhesion} = \frac{(raw\ sample\ adhesion - blank)}{avg(raw\ control\ adhesion - blank)}$$

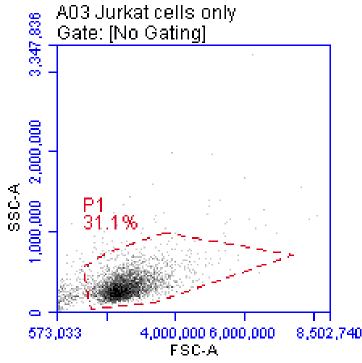

Figure 2.10: FSC vs SSC for Jurkat cells (untreated).

The raw plot from flow cytometer C6 with forward scattering against sideward scattering for Jurkat cells only.

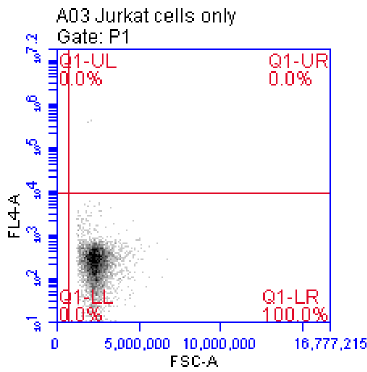

Figure 2.11: FSC vs FL4 for Jurkat cells (untreated).

The raw plot from flow cytometer C6 with forward scattering against fluorescence channel for Jurkat cells only. Gated in P1 which is cell positions.

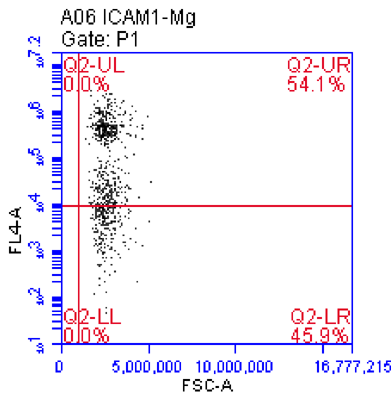

Figure 2.12: FSC vs FL4 for Jurkat cells with ICAM-1 beads.

The raw plot from flow cytometer C6 with forward scattering against fluorescence channel for an analyzed sample. Gated in P1 which is cell positions.

2.4.4 Inhibitors and reagents

For treatment of cells with BIRT377 (R&D Systems) and Cariporide (CAR, R&D Systems), the compounds were first dissolved in DMSO and diluted into HBS buffer (0.01% final concentration of DMSO). Antibody TS1/22 (3.6 µg mL^{-1}, ThermoFisher) was diluted in MQ H_2O before cell treatment. Inhibitors of hNEU enzymes were prepared as previously reported, and all compounds were used as the free carboxylate.[64,65,82,97] Zanamivir (ZAN) was obtained from **Biosynth** and used as provided. Lectin stains used were diCBM40-Alexa Fluor647 (2 µg mL^{-1}; a generous gift of Dr. L Mahal) and PNA-Alexa Fluor647 (5 µg mL^{-1}; ThermoFisher). Cells were incubated with the lectins on ice in the dark for 30 min and analyzed by flow cytometry.

Oseltamivir carboxylate (OC) was prepared by hydrolysis of oseltamivir (Biosynth Carbosynth). A sample of oseltamivir (20 mg, 0.05 mmol) was dissolved in methanol, and potassium carbonate was added (96.6 mg, 0.7 mmol). The reaction was stirred at rt for 2h. After completion, the solution was neutralized with Amberlite IR 120 (H^+). The suspension was left stirring for 30 min at room temperature, followed by filtration. The filtrate was concentrated under vacuum, and then the product was purified by reverse-phase chromatography to give OC.

Chapter III

Detection of neuraminidase activity in human blood[1]

[1]In this project, serum and plasma samples from healthy donors were obtained from the West laboratory (University of Alberta) or from samples used in Chapter II for PBMC isolation. Inhibitor compounds used in this chapter were prepared by Drs. Elisa Garcia Carvajal and Mostafa Radwan.

3.1 Introduction

Glycosylation is a common post-translational modification that adds specific glycan residues to proteins and lipids. The appended glycans may regulate the function and stability of the resulting glycoconjugate.[29] Glycans play critical roles in fundamental biological processes including cell signaling, cell-cell adhesion, and recognition.[29] Glycosylation has been linked to disease progression in cancer, where it contributes to metastasis, cell-cell communication, signaling, and aberrant adhesion.[98] Cancer cells are often observed to express abnormal glycosylation patterns, suggesting that modulation of glycosylation could be a potential therapeutic strategy.[99,100]

Biosynthesis of glycans is complex, though most structures are produced as proteins transit the endoplasmic reticulum and Golgi compartments.[101] Numerous enzymes participate in the regulation of glycan structures, including enzymes that may add or remove functionality.[102] Glycosidase enzymes that remove residues from glycans are important in biosynthesis but may also affect glycan structures at the cell membrane or even in circulation. Neuraminidase enzymes (NEU) are exo-glycosidases that trim terminal N-acetyl-neuraminic acids (Neu5Ac, or sialic acid) from glycolipids and glycoproteins. There are four isoenzymes of human neuraminidase (hNEU) isoenzymes: NEU1, NEU2, NEU3, and NEU4.[44] These enzymes are found in distinct cellular compartments, and the isoenzymes have specific substrate preferences.[47] For example, NEU1 is localized in lysosomes where it participates in glycoprotein degradation; while NEU3 is found in lysosomes and the plasma membrane where it modifies glycolipid substrates.[47] NEU modulate the sialylation of tumor-associated glycoproteins, and have been implicated in malignancy, oncogenesis, invasiveness, and metastasis.[49,51,60] For

instance, NEU3 is up-regulated in colon, renal, and prostate cancers, where it suppresses apoptosis of cancer cells.[103–105] In contrast, NEU1 and NEU4 are suggested to act as tumor suppressors, involved in regulating cell adhesion and invasiveness.[106,107] Both NEU1 and NEU4 are down-regulated in colon cancer.[106,107]

Serum is a key component of blood that circulates and transports cells and molecules throughout the body. Serum proteins are often used as biomarkers that report on the physiological state of the body, forming the basis of many diagnostic tests for disease progression.[108] The amount of sialic acid on specific protein biomarkers has been proposed as a biomarker in cancer.[109] Previous studies have found that NEU enzymes are found in human serum; therefore, these enzymes could regulate glycoprotein biomarkers or may serve as biomarkers themselves. Hata et al. observed that NEU1 and NEU3 are the dominant contributors to serum NEU activity.[57] The levels of different hNEU isoenzymes in serum have been suggested to vary depending on cancer progression. For example, NEU3 is highly overexpressed in the serum of prostate cancer patients compared to healthy individuals;[57] whereas, NEU1 overexpression in colon cancers suppresses metastasis.[106] This suggests that NEU isoenzymes modulate glycosylation of different glycoconjugate targets, leading to distinct functions in cancer progression. Therefore, characterizing the activity of hNEU isoenzymes in serum may provide insights into cancer progression and could be used as diagnostics.

Although serum NEU activity has been known for some time,[110] only a few studies have examined the activity of specific hNEU isoenzymes in serum. Miyagi and co-workers identified NEU1 and NEU3 isoenzymes in human serum and analyzed changes in enzyme levels in cancer patients.[57] Studies of sepsis in murine models has

identified NEU1 in serum, and suggested that the enzyme may contribute to protein senescence.[111,112] The relationship between serum activity levels of hNEU isoenzymes and individual physiological conditions has not yet been fully characterized. Addressing this gap could open a door for cancer therapeutic approaches.

At the present time, there is no simple and direct method to detect the activity of individual hNEU isoenzymes. Typical assay approaches for NEU activity exploit fluorogenic substrate 4-methlumbelliferyl-*N*-acetyl-neuraminic acid (4MU-NANA).[90,113] Although 4MU-NANA is highly sensitive, it lacks specificity for individual hNEU isoenzymes, and its K_m values are provided in **Table 3.1**.[71] Our group previously developed selective inhibitors for specific isoenzymes.[62–64] We hypothesized that we could develop an assay for hNEU isoenzymes in serum by combining one or multiple of these selective inhibitors with a standard 4MU-NANA assay. By comparing assay results with and without inhibitors, we hoped to determine the relative activity of individual NEU isozymes in patient serum. This chapter will describe our work to develop a protocol for 4MU-NANA assays of human serum, and initial studies with inhibitors of hNEU isoenzymes.

hNEU isoenzymes	K_m (μM)
hNEU1	56 ± 18[71]
hNEU2	119 ± 21[71]
hNEU3	48 ± 9[71]
hNEU4	17 ± 2[71]

Table 3.1: K_m values of 4MU-NANA for hNEU isoenzymes.

Adapted from Richards, M. R.; Guo, T.; Hunter, C. D.; Cairo, C. W. Molecular Dynamics Simulations of Viral Neuraminidase Inhibitors with the Human Neuraminidase Enzymes: Insights into Isoenzyme Selectivity. *Bioorg. Med. Chem.* **2018**, *26* (19), 5349–5358.

3.2 Results & Discussion

3.2.1 Detection of NEU activity from frozen human serum

To identify NEU activity in human blood, we first compared frozen serum and plasma samples using a standard 4MU-NANA assay. As shown in **Table 3.2**, only serum samples double the original volume (20 μL of serum into 40 μL of final reaction volume, $\frac{1}{2}$ concentration) provided positive signals, while no detectable activity was observed in plasma samples. Since serum demonstrated higher hNEU activity than plasma, proceeded with serum samples for further assays.

Average Fluorescence Signals							
Serum #1 (1x)	**Serum #1 (2x)**	**Serum #2 (1x)**	**Serum #2 (2x)**	**Plasma #1 (1x)**	**Plasma #1 (2x)**	**Plasma #2 (1x)**	**Plasma #2 (2x)**
-6.562	41.34	-78.029	53.788	-129.35	-5.97	-122.57	-15.871

Table 3.2: Comparison of hNEU activity in human serum and plasma samples. Samples from two donors (#1 and #2) were used for 4MU-NANA assays to detect hNEU activity in 10 μL (1X volume, 10 μL of samples into 40 μL of final reaction volume, $\frac{1}{4}$ concentration) and 20 μL (2X volume, 20 μL of samples into 40 μL of final reaction volume, $\frac{1}{2}$ concentration) samples of serum and plasma after a 30 min incubation. 4 replicates were performed for each sample.

Since sample dilution was a key factor in the assay results, we next determined the optimal volume. Initially, we used 20 μL of serum (into 40 μL final reaction volume, $\frac{1}{2}$ concentration), increasing to 50 μL (into 60 μL final reaction volume, $\frac{5}{6}$ concentration)

showed improved results. However, we observed fluorescence signals in 50 μL of serum samples even without adding 4MU-NANA, suggesting interference from components in the serum. Human serum contains various substances, particularly albumin and IgG which are abundant and can contribute to signal interference. Additionally, Hata et al. proposed using smaller amounts of serum samples (2.5 to 5 μL) would improve hNEU activity detection.[57] Hata et al. found that amounts of more than 5 μL resulted in reduced activity for 4MU-NANA substrate, which they attributed to an unknown NEU inhibitors present in serum that interfered with the assay.[57] Based on their findings, we optimized our assay by reducing the serum volume used.

In **Figure 3.1**, we compared the effect of different serum volumes and incubation times on hNEU activity detection. There was no activity difference between 2.5 μL and 5 μL serum volumes with 30 min incubation and increasing incubation time from 30 to 60 min did not improve activity detection and even resulted in a slight reduction in signals. Increasing both serum volume and incubation time mildly boosted the detected signals, but it is not worth the minor improvement with the additional time and volume. Therefore, we determined that using 2.5 μL of serum with 30 min incubation in the 4MU-NANA assay provided the most reliable results. After confirming hNEU activity in serum, we proceeded to assess the activity differences of hNEU isoenzymes by employing selective inhibitors of hNEU.

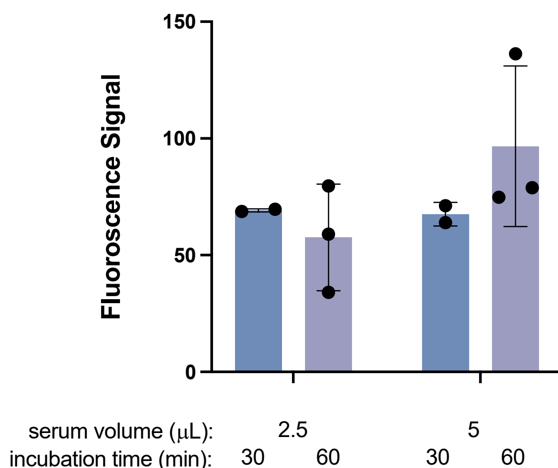

Figure 3.1: Confirmation of hNEU activity in frozen human serum.

hNEU activity was tested with in 2.5 μL (into 12.5 μL final volume, $\frac{1}{5}$ concentration) and 5 μL (into 20 μL final volume, $\frac{1}{4}$ concentration) of frozen serum with different incubation times using the 4MU-NANA assay, 2–3 replicates were performed for each condition.

3.2.2 Inhibition of isoenzyme activity in frozen serum

To identify activity differences between hNEU isoenzymes in serum, we pre-treated the serum with selective inhibitors and measured fluorescence signals using the 4MU-NANA assay. NanI is a neuraminidase from *Clostridium perfringens* which can cleave terminal sialic acid residues from glycoproteins and glycolipids.[114] It was used to plot a standard calibration curve in **Figure 3.2a** to convert 4MU fluorescence signal into neuraminidase activity. Shown in **Figure 3.2b** is the activity of NEU in the presence of NEU isoenzyme-selective inhibitors (10 μM; **Table 3.3**). DANA is a non-selective inhibitor that suppress all hNEU activities.[71] After treatment with DANA, serum hNEU activity showed a slight decrease. This confirms the presence of detectable hNEU

activity in serum. CG33300 and CG25900 are selective inhibitors for hNEU1.[64] Treatment with the NEU1 inhibitor CG33300 caused a significant reduction in serum activity. In contrast, a weaker NEU1 inhibitor, CG25900, did not show significant inhibition. This discrepancy is likely a reflection of the lower potency of CG25900. We did not observe any changes in NEU activity after treatment with the NEU4 inhibitor CY16600.[70] Treatment with NEU3 inhibitors CR96300 and CG22600 did not show significant differences from controls. Compound CG22600 required the addition of cyclodextrin to the buffer, and these results were compared to a cyclodextrin control. We considered that the native inhibitory effect of serum noted by Hata et al. could be preventing accurate measures of NEU3 activity.[57] Testing of CR96300 at a lower concentration (0.1 μM) showed significant suppression of NEU activity in serum (**Figure 3.3**). NEU3 is reported to have significant expression in human serum.[57] The 4MU-NANA inhibition assay results here are consistent with NEU3 and NEU1 activity in serum. However, we observed a dramatic effect of serum dilution and unexpected dependance on inhibitor concentrations that will require further studies to obtain consistent results.

Compound	Target(s)	IC_{50} [μM]
DANA	NEU1-4	1.6 – 25[71]
CG25900	NEU1	0.42 ± 0.06[64]
CG33300	NEU1	0.14 ± 0.01[64]
CR96300	NEU3	0.31 ± 0.07[65]
CG22600	NEU3	0.58 ± 0.14[63]
CY16600	NEU4	0.16 ± 0.01[70]

Table 3.3: Previously determined activity of hNEU inhibitors used in this study.

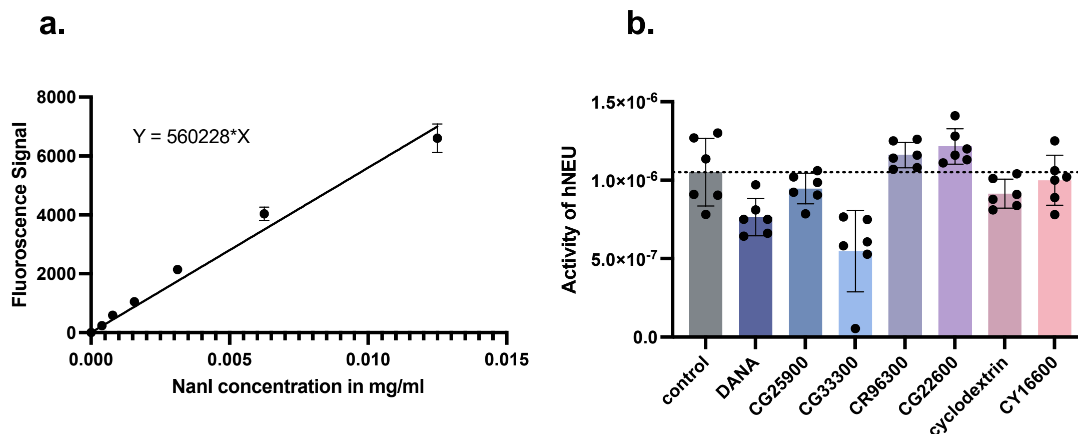

Figure 3.2: NEU activity in human serum.

(**a**) Standard calibration curve for NanI activity. (**b**) 4MU-NANA signal from serum after pre-treatment with isoenzyme-specific inhibitors of NEU. Human serum was pre-treated with 10 μM of hNEU inhibitors (CG22600 was dissolved in cyclodextrin), and 4MU-NANA assay was used to measure fluorescence signals. To quantify activity of hNEU, the NanI standard calibration curve was applied to convert fluorescence signals into NEU activity. 6 replicates are shown for each treatment. Data were compared by one-way ANOVA (*, $p \leq 0.05$; ****, $p < 0.0001$). Error bars represent standard deviation.

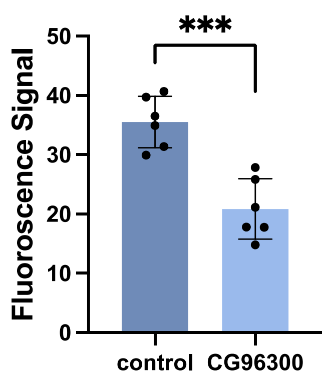

Figure 3.3: Identification of hNEU3 in human serum.

Human serum was treated with low concentration of 0.1 μM CR96300, and 4MU-NANA assay was used to measure fluorescence signals. 6 replicates were performed for each condition, and the results were compared by a Student's t-test (***, $p \leq 0.0005$). Error bars represent standard deviation.

3.2.3 Activity of DANA in frozen serum

We expected a dose-dependent effect of NEU inhibitors on serum samples, however results with lower concentrations of CR96300 suggested an unusual concentration dependence. Therefore, we tested the activity of serum NEU over a range of concentration for DANA (**Figure 3.4**). DANA is a non-selective inhibitor of hNEU. The results of the DANA titration were relatively noisy and may suggest moderate inhibition of NEU activity close to 10 μM. The relatively small shift in activity (55% to 40%) may indicate that the conditions require additional optimization. Similar titrations of NEU1 and NEU3 inhibitors CG33300 and CR96300 showed no major concentration-dependent effects (data not shown). Based on the small effects of inhibitors in these assays and the variability seen with dilution of frozen serum samples, we next decided to test hNEU activity from fresh serum.

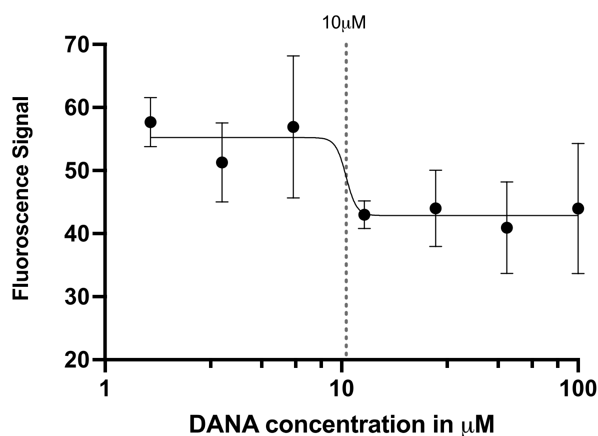

Figure 3.4: NEU activity from serum treated with DANA.

The serum sample was treated with increasing concentrations of DANA, and fluorescence signals were measured using the 4MU-NANA assay. 4 replicates were performed for each concentration. Outliers in the data were removed using the ROUT method, and an IC_{50} inhibition model was applied for analysis.

3.2.4 Detection of hNEU activity in fresh plasma

Serum is the soluble portion of whole blood after removal of cells and coagulating protein components. Plasma is the soluble portion of whole blood after removal of cells, but still contains coagulating protein components. In our studies of PBMCs for another project (see Chapter II) we generated diluted plasma samples that were treated with citrate anti-coagulant. We decided to test these plasma samples to test if hNEU activity was higher in fresh samples. Results of 4MU-NANA assays with fresh plasma samples are shown in **Figure 3.5.** Unfortunately, we observed negligible activity in the samples tested along with high variability between replicates. It is possible that these results could be improved through optimization of the dilution factor (vide supra).

Further optimization of the assay will be required to obtain consistent results for hNEU activity in fresh plasma samples.

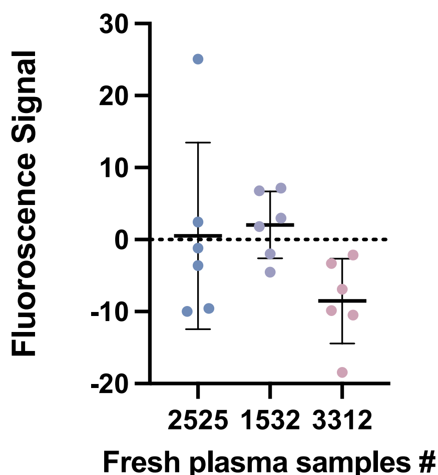

Figure 3.5: NEU activity detected in fresh plasma.

4MU-NANA assay was used to measure fluorescence signals of 2.5 µL of fresh plasma and PBS (used as a blank) with 30 min incubation. The data presented in this table were corrected by subtracting the blank controls, with 6 replicates performed for each biological sample. The data were compared by one-way ANOVA which showed NS ($p>0.05$).

3.3 Conclusion

In this study, we tested conditions for analysis of NEU activity in frozen serum and fresh plasma samples. We found that NEU activity in frozen serum samples was dependent on the amount and dilution of the sample in the assay condition. The optimal serum volume for the 4MU-NANA assay was 2.5 µL, with 30 min incubation. This activity could be partially inhibited by compounds that block NEU1 and NEU3 enzyme

activity. However, some inhibitors exhibited unusual concentration-dependent effects with our NEU3 inhibitor CR96300 being more active at lower concentrations. Attempts to compare NEU activity in fresh plasma samples to frozen serum were unsuccessful, and further optimization will be required to confirm such differences.

Future studies will be required to optimize the NEU activity assays in serum. A more complete study of hNEU activity that compares fresh and frozen serum directly will be required. These studies will need to analyze any dependence on concentration of the serum sample due to the inhibitor effects of serum components. Once optimized, the assay should then be used to test the effects of hNEU inhibitors to see if more accurate inhibition curves can be determined for individual inhibitors. These methods could then be used to confirm the identity of serum NEU isoenzymes and their sensitivity to inhibitors.

3.4 Materials & Methods

3.4.1 Frozen serum and plasma samples

Serum samples from healthy donors were obtained from the West laboratory (University of Alberta) and stored at -20 °C. Samples were thawed at room temperature immediately before use.

3.4.2 Preparation of fresh plasma samples

Fresh plasma samples were isolated from 7 – 8 mL whole blood donated by healthy individuals (University of Alberta, HREB Protocol #Pro0016491). The whole blood was diluted with sterile PBS at a 1:1 ratio (resulting in total volume of

approximately 15 mL). Next, the diluted blood was gently overlaid onto 10 mL of Histopaque®-1077 (ficoll density gradient medium, 1.077 g mL^{-1}, Catalog# 10771-100ML, Sigma-Aldrich) for density gradient centrifugation at 1000 rcf for 20 min. The clear plasma layer was transferred into a new tube, and NEU activity was immediately tested using 4MU-NANA assay.

3.4.3 Serum 4MU-NANA assay

Serum samples (2.5 µL) were incubated with reaction buffer (5 µL, pH 4.5, 0.1 M NaOAc) and 4MU-NANA (5 µL of 0.4 mM) at 37 °C for 30 min. After incubation, quenching buffer was added (62.5 µL, pH 10.7, 0.2 M NaOH/Glycine) to stop the reactions. The reaction mixture (75 µL) was transferred into a black 96-well plate. Then fluorescence signals were measured to determine NEU activity with a fluorescent plate reader (excitation, 365 nm; emission, 445 nm).

3.4.4 Serum 4MU-NANA assay with inhibitors

To identify activity differences between serum isoenzymes, we applied selective inhibitors for NEU1, NEU3, and NEU4 to serum samples. First, serum samples (2.5 µL) were pre-incubated with reaction buffer (5 µL, pH 4.5, 0.1 M NaOAc) and inhibitors (5 µL, final concentration of 10 µM) on ice for 15 min. Next, 4MU-NANA was added (5 µL of 0.4 mM) and the reaction was incubated at 37 °C for 30 min. After incubation, quenching buffer (87.5 µL, pH 10.7, 0.2 M NaOH/Glycine) was added to stop the reaction. of The reaction mixture (75 µL) was transferred into a black 96-well plates. Then fluorescence signals were measured to determine NEU activity with a fluorescent plate reader (excitation, 365 nm; emission, 445 nm).

3.4.5 4MU-NANA assay standard curve

NanI (neuraminidase from *Clostridium Perfringens*, Catalog# N2876-25UN, 4.1 units mg^{-1}, Sigma Aldrich) was diluted by MQ H_2O to generate five different concentrations (0.0125, 0.00625, 0.003125, 0.001563, and 0.000781 mg mL^{-1}). Each NanI concentration (10 µL) was incubated with reaction buffer (20 µL, pH 5.6, 0.1 M NaOAc) and 4MU-NANA (10 µL of 0.4 mM) at 37 ℃ for 30 min. After incubation, quenching buffer (200 µL, pH 10.7, 0.2 M NaOH/Glycine) was added to stop the reaction. Then fluorescence signals were measured to determine the NanI activity using a fluorescence plate reader (excitation, 365 nm; emission, 445 nm).

3.4.6 IC_{50} determinations of NEU inhibitors

Serum samples (2.5 µL) were pre-incubated with reaction buffer (5 µL, pH 4.5, 0.1 M NaOAc) and inhibitors (5 µL, final concentration from 100 µM to 1.56 µM) on ice for 15 min. Next, 4MU-NANA was added (5 µL of 0.4 mM) and the reaction was incubated at 37 ℃ for 30 min. After incubation, quenching buffer (87.5 µL, pH 10.7, 0.2 M NaOH/Glycine) was added to stop the reaction. The reaction mixture (75 µL) was transferred into a black 96-well plates. Then fluorescence signals were measured to determine NEU activity with a fluorescent plate reader (excitation, 365 nm; emission, 445 nm).

Chapter IV

Conclusions and future directions

4.1 Thesis overview

In this thesis, we investigated the hypothesis that the glycan structure of LFA-1 regulates its affinity and binding to ICAM-1. To test if individual NEU isoenzymes contribute differently to this regulatory mechanism, we used pharmacological inhibitors developed previously in our group. Chapter II describes our investigation of native hNEU enzyme effects on LFA-1-ICAM-1 adhesion. By pretreating cells with selective hNEU inhibitors and a flow cytometry-based assay we compared the effects of individual isoenzymes on this process. The results demonstrated that hNEU act as negative regulators of LFA-1 adhesion in leukocytes. Among the hNEU isoenzymes, hNEU1 and hNEU4 were found to play suppressive roles in both resting and activated LFA-1 adhesion, whereas hNEU3 exhibited a negative regulation effect in the resting state. These findings suggest that selective inhibitors of hNEU isoenzymes have the potential as anti-inflammatory drugs, providing a new therapeutic approach for modulating inflammatory responses.

Previous studies have suggested the hNEU are found circulating in blood. However, no current methods allow for easy detection of specific hNEU isoenzyme activity in these samples. In Chapter III, we attempted to develop a detection strategy for hNEU isoform activity in human serum. We developed a straightforward method to measure serum hNEU activity using the 4MU-NANA assay with selective inhibitors. Our results are consistent with the presence of hNEU1 and hNEU3 in blood, while hNEU4 is likely negligible. In this study, we identified several issues: 1. The activity of hNEU is unstable in frozen serum samples and degrades over time. 2. There are interferences in serum samples that can affect the results during the 4MU-NANA

detection assay. For instance, unknown hNEU inhibitors in the serum interfere with the inhibition assay, while certain serum components also cause fluorescence interference with 4MU-NANA signals. 3. The activity of hNEU is more dominant in serum than in plasma.

Overall, hNEU play critical roles in LFA-1 adhesion and the inflammatory cascade. Although the mechanisms are not fully characterized, this study suggests that selective inhibitors of hNEU represent a promising therapeutic approach for treating inflammation and chronic inflammation associated diseases. Furthermore, the activity of serum hNEU could act as a biomarker or indicator for the early diagnosis of cancer and other diseases.

4.2 Future directions

In this study, we investigated the roles of hNEU isoenzymes in LFA-1–ICAM-1 adhesion in T cell and PBMC models, testing both resting and activated states of LFA-1. Additionally, we confirmed the presence of hNEU1 and hNEU3 in human serum. However, several aspects remain uncharacterized, and important questions still need to be addressed.

4.2.1 The targets of hNEU in LFA-1 mediated leukocyte adhesion

In Chapter II, we demonstrated that hNEU negatively regulates LFA-1 adhesion in leukocytes, and hNEU did alter cell surface glycosylation. However, the lectin staining results reflected overall glycosylation levels on the cell surface, rather than specifically identifying changes in adhesion receptors or other molecules involved in LFA-1 adhesion. Our group previously proposed that hNEU may regulate LFA-1

adhesion either by directly modifying adhesion receptors or by altering the composition of membrane glycolipids.[35] Therefore, future investigations should aim to identify the specific hNEU targets that regulate LFA-1–ICAM-1 adhesion. Notably, diCBM40 can recognize both α 2-3 and α 2-6 linked sialic acids, though it exhibits higher affinity for α 2-3 linkages,[115] which are predominantly found on glycolipids. In contrast, sambucus nigra agglutinin (SNA) preferentially binds α 2-6 linked sialosides that are mostly present on glycoproteins. Thus, by comparing diCBM40 and SNA staining after hNEU inhibitor treatment, we can determine whether the targets of hNEU are more likely glycolipids or glycoproteins.

4.2.2 The on-target effect of hNEU inhibitors

In Chapter II, we displayed the effects of hNEU selective inhibitors on LFA-1–ICAM-1 adhesion. Our previous studies have shown that these selective inhibitors (CG33300, CR96300, CY16600) exhibit highly selectivity for specific hNEU isoenzymes, with favorable K_i values.[64,65,70] However, the possibility of off-target effects cannot be excluded. To address this, we propose using NEUx KO cells treated with and without their corresponding selective inhibitors. If the inhibitors are truly on-target, treatment of NEUx KO cells should result in no observable effect.

4.2.3 The roles of hNEU in LFA-1 adhesion in vivo

In this study, we identified the negative effects of hNEU isoenzymes on LFA-1 adhesion in vitro. However, it remains unclear from our study if these systems behave the same in vivo. Murine KO models of hNEU isoenzymes are known,[55,116] and these models could help to confirm the roles of hNEU in LFA-1 adhesion. Previous studies

have found reduced leukocyte recruitment in NEU1 and NEU3 KO animals, but there could be multiple mechanisms responsible for this observation. For example, adhesion assays similar to the cytometry assay in Chapter II could be used on splenocytes from KO animals to determine if there are effects on LFA-1 adhesion. This approach would allow direct observation of hNEU-mediated regulation of LFA-1 adhesion within the context of inflammation and immune responses.

Furthermore, to assess the effects of hNEU selective inhibitors in vivo, we propose an experimental approach with inducing inflammation firstly, followed by treatment with hNEU selective inhibitors. Subsequent determination of the number of leukocytes that reach the inflammation sites could support our model. In a previous study, Howlader et al. conducted a similar experiment but used different hNEU inhibitors, yielding inconclusive results. This may be due to the potency of those inhibitors or the presence of additional in vivo mechanisms that limited the effectiveness of hNEU inhibition. Based on our findings, hNEU selective inhibitor treatments enhance LFA-1 adhesion, a key step in leukocyte recruitment. Hence, we expect that inhibiting hNEU will increase the number of leukocytes at inflammation sites compared with non-treated groups. This in vivo experiment directly presents the potential of hNEU selective inhibitor treatment as an anti-inflammatory therapy.

4.2.4 Quantifying the activity of individual hNEU isoenzymes in human serum

In Chapter III, we attempted to develop a detection assay for serum hNEU activity. Our results were consistent with the presence of NEU1 and NEU3 in human blood. However, we have not quantified the individual hNEU isoenzyme activities in

serum. In future studies, as the detection assay is further optimized, we aim to determine accurate inhibition curves and quantify the serum activity of each hNEU isoenzyme.

4.2.5 The roles of serum hNEU isoenzymes in cancer patients

Currently, more studies have recognized the critical roles of hNEU in cancer, with their expression levels being associated with disease progression. Some studies have suggested that hNEU1 and hNEU4 are down-regulated in cancers,[106,107] while hNEU3 is overexpressed[103,104]. However, these studies primarily focus on the expression levels of hNEU in tissues or cells, with absence of studies on detecting hNEU activity in blood serum.

Thus, we aim to characterize the activity of serum hNEU isoenzymes in healthy individuals and compare their expression levels in cancer patients. A decade ago, K. Hata's study demonstrated that prostate cancer patients overexpress hNEU3 in serum using the ELISA method.[57] However, the ELISA method is time-consuming, requiring several days for coating, blocking, incubation, washing, and visualization, which limits its immediate application. Therefore, using the 4MU-NANA assay with selective hNEU inhibitors to directly detect serum activity of hNEU isoenzymes in cancer patients could provide a promising and quick diagnostic approach for cancer.

References

(1) Buckley, R. H. Molecular Defects in Human Severe Combined Immunodeficiency and Approaches to Immune Reconstitution. *Annu. Rev. Immunol.* **2004**, *22* (1), 625–655. https://doi.org/10.1146/annurev.immunol.22.012703.104614.

(2) Medzhitov, R. Origin and Physiological Roles of Inflammation. *Nature* **2008**, *454* (7203), 428–435. https://doi.org/10.1038/nature07201.

(3) Nathan, C.; Ding, A. Nonresolving Inflammation. *Cell* **2010**, *140* (6), 871–882. https://doi.org/10.1016/j.cell.2010.02.029.

(4) Hotamisligil, G. S. Inflammation and Metabolic Disorders. *Nature* **2006**, *444* (7121), 860–867. https://doi.org/10.1038/nature05485.

(5) Libby, P.; Ridker, P. M.; Maseri, A. Inflammation and Atherosclerosis. *Circulation* **2002**, *105* (9), 1135–1143. https://doi.org/10.1161/hc0902.104353.

(6) Calder, P. C.; Albers, R.; Antoine, J.-M.; Blum, S.; Bourdet-Sicard, R.; Ferns, G. A.; Folkerts, G.; Friedmann, P. S.; Frost, G. S.; Guarner, F.; Løvik, M.; Macfarlane, S.; Meyer, P. D.; M'Rabet, L.; Serafini, M.; Van Eden, W.; Van Loo, J.; Vas Dias, W.; Vidry, S.; Winklhofer-Roob, B. M.; Zhao, J. Inflammatory Disease Processes and Interactions with Nutrition. *Br. J. Nutr.* **2009**, *101* (S1), 1–45. https://doi.org/10.1017/S0007114509377867.

(7) Leick, M.; Azcutia, V.; Newton, G.; Luscinskas, F. W. Leukocyte Recruitment in Inflammation: Basic Concepts and New Mechanistic Insights Based on New Models and Microscopic Imaging Technologies. *Cell Tissue Res.* **2014**, *355* (3), 647–656. https://doi.org/10.1007/s00441-014-1809-9.

(8) Murphy, K.; Weaver, C. *Janeway's Immunobiology*, 9th edition.; Garland Science/Taylor & Francis Group, LLC: New York, NY, 2016.

(9) Zola, H. CD Molecules 2005: Human Cell Differentiation Molecules. *Blood* **2005**, *106* (9), 3123–3126. https://doi.org/10.1182/blood-2005-03-1338.

(10) Kuijpers, T. W.; Verhoeven, A. J.; Roos, D. Leukocyte Adhesion Deficiency Type 1 (LAD-1)/Variant. *J. Clin. Invest.* **1997**, *100* (7), 1725–1733.

(11) Fekadu, J.; Modlich, U.; Bader, P.; Bakhtiar, S. Understanding the Role of LFA-1 in Leukocyte Adhesion Deficiency Type I (LAD I): Moving towards Inflammation? *Int. J. Mol. Sci.* **2022**, *23* (7), 3578. https://doi.org/10.3390/ijms23073578.

(12) Kubes, P.; Ward, P. A. Leukocyte Recruitment and the Acute Inflammatory Response. *Brain Pathol.* **2000**, *10* (1), 127–135. https://doi.org/10.1111/j.1750-3639.2000.tb00249.x.

(13) Ley, K.; Laudanna, C.; Cybulsky, M. I.; Nourshargh, S. Getting to the Site of Inflammation: The Leukocyte Adhesion Cascade Updated. *Nat. Rev. Immunol.* **2007**, *7* (9), 678–689. https://doi.org/10.1038/nri2156.

(14) Vestweber, D. How Leukocytes Cross the Vascular Endothelium. *Nat. Rev. Immunol.* **2015**, *15* (11), 692–704. https://doi.org/10.1038/nri3908.

(15) McEver, R. P. Selectins: Initiators of Leucocyte Adhesion and Signalling at the Vascular Wall. *Cardiovasc. Res.* **2015**, *107* (3), 331–339. https://doi.org/10.1093/cvr/cvv154.

(16) Cummings, R. D.; Chiffoleau, E.; Van Kooyk, Y.; McEver, R. P. Chapter34 C-Type Lectins. In *Essentials of glycobiology*; Elsevier, 2010; Vol. 479, pp 223–241. https://doi.org/10.1016/S0076-6879(10)79013-3.
(17) McEver, R. P.; Zhu, C. Rolling Cell Adhesion. *Annu. Rev. Cell Dev. Biol.* **2010**, *26* (1), 363–396. https://doi.org/10.1146/annurev.cellbio.042308.113238.
(18) Jiang, S.; Li, H.; Zhang, L.; Mu, W.; Zhang, Y.; Chen, T.; Wu, J.; Tang, H.; Zheng, S.; Liu, Y.; Wu, Y.; Luo, X.; Xie, Y.; Ren, J. Generic Diagramming Platform (GDP): A Comprehensive Database of High-Quality Biomedical Graphics. *Nucleic Acids Res.* **2025**, *53* (D1), D1670–D1676. https://doi.org/10.1093/nar/gkae973.
(19) Kadry, Y. A.; Calderwood, D. A. Chapter 22: Structural and Signaling Functions of Integrins. In *Biochimica et Biophysica Acta (BBA) - Biomembranes*; 2020; Vol. 1862, p 183206.
(20) Kelly, M.; Hwang, J. M.; Kubes, P. Modulating Leukocyte Recruitment in Inflammation. *J. Allergy Clin. Immunol.* **2007**, *120* (1), 3–10. https://doi.org/10.1016/j.jaci.2007.05.017.
(21) Alon, R.; Dustin, M. L. Force as a Facilitator of Integrin Conformational Changes during Leukocyte Arrest on Blood Vessels and Antigen-Presenting Cells. *Immunity* **2007**, *26* (1), 17–27. https://doi.org/10.1016/j.immuni.2007.01.002.
(22) Van Kooyk, Y.; Figdor, C. G. Avidity Regulation of Integrins: The Driving Force in Leukocyte Adhesion. *Cell Biol.* **2000**, *12*, 542–547.
(23) Park, E. J.; Myint, P. K.; Ito, A.; Appiah, M. G.; Darkwah, S.; Kawamoto, E.; Shimaoka, M. Integrin-Ligand Interactions in Inflammation, Cancer, and Metabolic Disease: Insights Into the Multifaceted Roles of an Emerging Ligand Irisin. *Front. Cell Dev. Biol.* **2020**, *8*, 588066. https://doi.org/10.3389/fcell.2020.588066.
(24) Cairo, C. W.; Mirchev, R.; Golan, D. E. Cytoskeletal Regulation Couples LFA-1 Conformational Changes to Receptor Lateral Mobility and Clustering. *Immunity* **2006**, *25* (2), 297–308. https://doi.org/10.1016/j.immuni.2006.06.012.
(25) Shimaoka, M.; Xiao, T.; Liu, J.-H.; Yang, Y.; Dong, Y.; Jun, C.-D.; McCormack, A.; Zhang, R.; Joachimiak, A.; Takagi, J.; Wang, J.-H.; Springer, T. A. Structures of the alphaL I Domain and Its Complex with ICAM-1 Reveal a Shape-Shifting Pathway for Integrin Regulation.
(26) Lu, C.; Shimaoka, M.; Salas, A.; Springer, T. A. The Binding Sites for Competitive Antagonistic, Allosteric Antagonistic, and Agonistic Antibodies to the I Domain of Integrin LFA-11. *J. Immunol.* **2004**, *173* (6), 3972–3978. https://doi.org/10.4049/jimmunol.173.6.3972.
(27) Lu, X.; Lu, D.; Scully, M.; Kakkar, V. The Role of Integrins in Cancer and the Development of Anti-Integrin Therapeutic Agents for Cancer Therapy. *Perspect. Med. Chem.* **2008**, *2*, 57–73. https://doi.org/10.1177/1177391X0800200003.
(28) Varki, A. Biological Roles of Glycans. *Glycobiology* **2017**, *27* (1), 3–49. https://doi.org/10.1093/glycob/cww086.
(29) Reily, C.; Stewart, T. J.; Renfrow, M. B.; Novak, J. Glycosylation in Health and Disease. *Nat. Rev. Nephrol.* **2019**, *15* (6), 346–366. https://doi.org/10.1038/s41581-019-0129-4.

(30) Moremen, K. W.; Tiemeyer, M.; Nairn, A. V. Vertebrate Protein Glycosylation: Diversity, Synthesis and Function. *Nat. Rev. Mol. Cell Biol.* **2012**, *13* (7), 448–462. https://doi.org/10.1038/nrm3383.
(31) Sako, D.; Chang, X. J.; Barone, K. M.; Vachino, G.; White, H. M.; Shaw, G.; Veldman, G. M.; Bean, K. M.; Ahern, T. J.; Furie, B. Expression Cloning of a Functional Glycoprotein Ligand for P-Selectin. *Cell* **1993**, *75* (6), 1179–1186. https://doi.org/10.1016/0092-8674(93)90327-m.
(32) Van Kooyk, Y.; Rabinovich, G. A. Protein-Glycan Interactions in the Control of Innate and Adaptive Immune Responses. *Nat. Immunol.* **2008**, *9* (6), 593–601. https://doi.org/10.1038/ni.f.203.
(33) Janik, M. E.; Lityńska, A.; Vereecken, P. Cell Migration—The Role of Integrin Glycosylation. *Biochim. Biophys. Acta BBA - Gen. Subj.* **2010**, *1800* (6), 545–555. https://doi.org/10.1016/j.bbagen.2010.03.013.
(34) Gadhoum, S. Z.; Sackstein, R. CD15 Expression in Human Myeloid Cell Differentiation Is Regulated by Sialidase Activity. *Nat. Chem. Biol.* **2008**, *4* (12), 751–757. https://doi.org/10.1038/nchembio.116.
(35) Howlader, Md. A.; Li, C.; Zou, C.; Chakraberty, R.; Ebesoh, N.; Cairo, C. W. Neuraminidase-3 Is a Negative Regulator of LFA-1 Adhesion. *Front. Chem.* **2019**, *7*, 791. https://doi.org/10.3389/fchem.2019.00791.
(36) Pande, G. The Role of Membrane Lipids in Regulation of Integrin Functions. *Curr. Opin. Cell Biol.* **2000**, *12* (5), 569–574. https://doi.org/10.1016/S0955-0674(00)00133-2.
(37) Krauss, K.; Altevogt, P. Integrin Leukocyte Function-Associated Antigen-1-Mediated Cell Binding Can Be Activated by Clustering of Membrane Rafts. *J. Biol. Chem.* **1999**, *274* (52), 36921–36927. https://doi.org/10.1074/jbc.274.52.36921.
(38) Lietha, D.; Izard, T. Roles of Membrane Domains in Integrin-Mediated Cell Adhesion. *Int. J. Mol. Sci.* **2020**, *21* (15), 5531. https://doi.org/10.3390/ijms21155531.
(39) Sharma, D. K.; Brown, J. C.; Cheng, Z.; Holicky, E. L.; Marks, D. L.; Pagano, R. E. The Glycosphingolipid, Lactosylceramide, Regulates B1-Integrin Clustering and Endocytosis. *Cancer Res.* **2005**, *65* (18), 8233–8241. https://doi.org/10.1158/0008-5472.CAN-05-0803.
(40) Chatterjee, S.; Pandey, A. The Yin and Yang of Lactosylceramide Metabolism: Implications in Cell Function. *Biochim. Biophys. Acta BBA - Gen. Subj.* **2008**, *1780* (3), 370–382. https://doi.org/10.1016/j.bbagen.2007.08.010.
(41) Angata, T.; Varki, A. Chemical Diversity in the Sialic Acids and Related α-Keto Acids: An Evolutionary Perspective. *Chem. Rev.* **2002**, *102* (2), 439–470. https://doi.org/10.1021/cr000407m.
(42) Vestweber, D.; Blanks, J. E. Mechanisms That Regulate the Function of the Selectins and Their Ligands. *Physiol. Rev.* **1999**, *79* (1), 181–213. https://doi.org/10.1152/physrev.1999.79.1.181.
(43) Harduin-Lepers, A.; Mollicone, R.; Delannoy, P.; Oriol, R. The Animal Sialyltransferases and Sialyltransferase-Related Genes: A Phylogenetic Approach. *Glycobiology* **2005**, *15* (8), 805–817. https://doi.org/10.1093/glycob/cwi063.

(44) Miyagi, T.; Yamaguchi, K. Mammalian Sialidases: Physiological and Pathological Roles in Cellular Functions. *Glycobiology* **2012**, *22* (7), 880–896. https://doi.org/10.1093/glycob/cws057.
(45) MacDonald, E.; Forrester, A.; Valades-Cruz, C. A.; Madsen, T. D.; Hetmanski, J. H. R.; Dransart, E.; Ng, Y.; Godbole, R.; Shp, A. A.; Leconte, L.; Chambon, V.; Ghosh, D.; Pinet, A.; Bhatia, D.; Lombard, B.; Loew, D.; Larson, M. R.; Leffler, H.; Lefeber, D. J.; Clausen, H.; Caswell, P.; Shafaq-Zadah, M.; Mayor, S.; Weigert, R.; Wunder, C.; Johannes, L. Growth Factor-Induced Desialylation for the Fast Control of Endocytosis. September 13, 2023. https://doi.org/10.1101/2023.09.12.557183.
(46) Isaji, T.; Gu, J. Novel Regulatory Mechanisms of N-Glycan Sialylation: Implication of Integrin and Focal Adhesion Kinase in the Regulation. *Biochim. Biophys. Acta BBA - Gen. Subj.* **2024**, *1868* (6), 130617. https://doi.org/10.1016/j.bbagen.2024.130617.
(47) Miyagi, T. Mammalian Sialidases and Their Functions. *Trends Glycosci. Glycotechnol.* **2010**, *22* (125), 162–172. https://doi.org/10.4052/tigg.22.162.
(48) Smutova, V.; Albohy, A.; Pan, X.; Korchagina, E.; Miyagi, T.; Bovin, N.; Cairo, C. W.; Pshezhetsky, A. V. Structural Basis for Substrate Specificity of Mammalian Neuraminidases. *PLoS ONE* **2014**, *9* (9), e106320. https://doi.org/10.1371/journal.pone.0106320.
(49) Miyagi, T.; Takahashi, K.; Hata, K.; Shiozaki, K.; Yamaguchi, K. Sialidase Significance for Cancer Progression. *Glycoconj. J.* **2012**, *29* (8–9), 567–577. https://doi.org/10.1007/s10719-012-9394-1.
(50) Miyagi, T.; Wada, T.; Yamaguchi, K.; Hata, K. Sialidase and Malignancy: A Minireview. *Glycoconj. J.* **2003**, *20* (3), 189–198. https://doi.org/10.1023/B:GLYC.0000024250.48506.bf.
(51) Wada, T.; Hata, K.; Yamaguchi, K.; Shiozaki, K.; Koseki, K.; Moriya, S.; Miyagi, T. A Crucial Role of Plasma Membrane-Associated Sialidase in the Survival of Human Cancer Cells. *Oncogene* **2007**, *26* (17), 2483–2490. https://doi.org/10.1038/sj.onc.1210341.
(52) Cross, A. S.; Wright, D. G. Mobilization of Sialidase from Intracellular Stores to the Surface of Human Neutrophils and Its Role in Stimulated Adhesion Responses of These Cells. *J. Clin. Invest.* **1991**, *88* (6), 2067–2076. https://doi.org/10.1172/JCI115536.
(53) Bagriaçik, E.; Miller, K. Cell Surface Sialic Acid and the Regulation of Immune Cell Interactions: The Neuraminidase Effect Reconsidered. *Glycobiology* **1999**, *9* (3), 267–275.
(54) Sakarya, S.; Rifat, S.; Zhou, J.; Bannerman, D. D.; Stamatos, N. M.; Cross, A. S.; Goldblum, S. E. Mobilization of Neutrophil Sialidase Activity Desialylates the Pulmonary Vascular Endothelial Surface and Increases Resting Neutrophil Adhesion to and Migration across the Endothelium. *Glycobiology* **2004**, *14* (6), 481–494. https://doi.org/10.1093/glycob/cwh065.
(55) Howlader, Md. A.; Demina, E. P.; Samarani, S.; Guo, T.; Caillon, A.; Ahmad, A.; Pshezhetsky, A. V.; Cairo, C. W. The Janus-like Role of Neuraminidase Isoenzymes in Inflammation. *FASEB J.* **2022**, *36* (5), e22285. https://doi.org/10.1096/fj.202101218R.

(56) Howlader, Md. A.; Guo, T.; Cairo, C. W. Inhibitors of Human Neuraminidase Enzymes Block Transmigration in Vitro. *Front. Mol. Biosci.* **2022**, *9*, 835757. https://doi.org/10.3389/fmolb.2022.835757.

(57) Hata, K.; Tochigi, T.; Sato, I.; Kawamura, S.; Shiozaki, K.; Wada, T.; Takahashi, K.; Moriya, S.; Yamaguchi, K.; Hosono, M.; Miyagi, T. Increased Sialidase Activity in Serum of Cancer Patients: Identification of Sialidase and Inhibitor Activities in Human Serum. *Cancer Sci.* **2015**, *106* (4), 383–389. https://doi.org/10.1111/cas.12627.

(58) Cairo, C. W. Inhibitors of the Human Neuraminidase Enzymes. *MedChemComm* **2014**, *5* (8), 1067–1074. https://doi.org/10.1039/C4MD00089G.

(59) Miyagi, T.; Wada, T.; Yamaguchi, K.; Shiozaki, K.; Sato, I.; Kakugawa, Y.; Yamanami, H.; Fujiya, T. Human Sialidase as a Cancer Marker. *PROTEOMICS* **2008**, *8* (16), 3303–3311. https://doi.org/10.1002/pmic.200800248.

(60) Miyagi, T. Aberrant Expression of Sialidase and Cancer Progression. *Proc. Jpn. Acad. Ser. B* **2008**, *84* (10), 407–418. https://doi.org/10.2183/pjab.84.407.

(61) Glanz, V. Y.; Kashirskikh, D. A.; Grechko, A. V.; Yet, S.-F.; Sobenin, I. A.; Orekhov, A. N. Sialidase Activity in Human Blood Serum Has a Distinct Seasonal Pattern: A Pilot Study. *Biology* **2020**, *9* (8), 184. https://doi.org/10.3390/biology9080184.

(62) Cairo, C. W. Inhibitors of the Human Neuraminidase Enzymes. *MedChemComm* **2014**, *5* (8), 1067–1074. https://doi.org/10.1039/C4MD00089G.

(63) Guo, T.; Dätwyler, P.; Demina, E.; Richards, M. R.; Ge, P.; Zou, C.; Zheng, R.; Fougerat, A.; Pshezhetsky, A. V.; Ernst, B.; Cairo, C. W. Selective Inhibitors of Human Neuraminidase 3. *J. Med. Chem.* **2018**, *61* (5), 1990–2008. https://doi.org/10.1021/acs.jmedchem.7b01574.

(64) Guo, T.; Héon-Roberts, R.; Zou, C.; Zheng, R.; Pshezhetsky, A. V.; Cairo, C. W. Selective Inhibitors of Human Neuraminidase 1 (NEU1). *J. Med. Chem.* **2018**, *61* (24), 11261–11279. https://doi.org/10.1021/acs.jmedchem.8b01411.

(65) Radwan, M.; Guo, T.; Carvajal, E. G.; Bekkema, B. A. R.; Cairo, C. W. Bioisosteres at C9 of 2-Deoxy-2,3-Didehydro-N -Acetyl Neuraminic Acid Identify Selective Inhibitors of NEU3. *J. Med. Chem.* **2024**, *67* (16), 13594–13603. https://doi.org/10.1021/acs.jmedchem.3c02186.

(66) Demina, Ekaterina P.; Smutova, Victoria; Pan, Xuefang; Fougerat, Anne; Guo, Tianlin; Zou, Chunxia; Chakraberty, Radhika; Snarr, Brendan D.; Shiao, Tze C.; Rene, R.; Orekhov, Alexander N.; Miyagi, Taeko; Laffargue, Muriel; Sheppard, Donald C.; Cairo, Christopher W.; Pshezhetsky, Alexey V. Neuraminidases 1 and 3 Trigger Atherosclerosis by Desialylating Low-Density Lipoproteins and Increasing Their Uptake by Macrophages. *J. Am. Heart Assoc.* **2021**, *10* (4), e018756. https://doi.org/10.1161/JAHA.120.018756.

(67) Tran, H.-T. T.; Li, C.; Chakraberty, R.; Cairo, C. W. NEU1 and NEU3 Enzymes Alter CD22 Organization on B Cells. *Biophys. Rep.* **2022**, *2* (3), 100064. https://doi.org/10.1016/j.bpr.2022.100064.

(68) Howlader, M. A.; Guo, T.; Chakraberty, R.; Cairo, C. W. Isoenzyme-Selective Inhibitors of Human Neuraminidases Reveal Distinct Effects on Cell Migration. *ACS Chem. Biol.* **2020**, *15* (6), 1328–1339. https://doi.org/10.1021/acschembio.9b00975.

(69) Silvestri, I.; Testa, F.; Zappasodi, R.; Cairo, C. W.; Zhang, Y.; Lupo, B.; Galli, R.; Di Nicola, M.; Venerando, B.; Tringali, C. Sialidase NEU4 Is Involved in Glioblastoma Stem Cell Survival. *Cell Death Dis.* **2014**, *5* (8), e1381. https://doi.org/10.1038/cddis.2014.349.

(70) Albohy, A.; Zhang, Y.; Smutova, V.; Pshezhetsky, A. V.; Cairo, C. W. Identification of Selective Nanomolar Inhibitors of the Human Neuraminidase, NEU4. *ACS Med. Chem. Lett.* **2013**, *4* (6), 532–537. https://doi.org/10.1021/ml400080t.

(71) Richards, M. R.; Guo, T.; Hunter, C. D.; Cairo, C. W. Molecular Dynamics Simulations of Viral Neuraminidase Inhibitors with the Human Neuraminidase Enzymes: Insights into Isoenzyme Selectivity. *Bioorg. Med. Chem.* **2018**, *26* (19), 5349–5358. https://doi.org/10.1016/j.bmc.2018.05.035.

(72) Rudd, P. M.; Wormald, M. R.; Stanfield, R. L.; Huang, M.; Mattsson, N.; Speir, J. A.; DiGennaro, J. A.; Fetrow, J. S.; Dwek, R. A.; Wilson, I. A. Roles for Glycosylation of Cell Surface Receptors Involved in Cellular Immune Recognition. *J. Mol. Biol.* **1999**, *293* (2), 351–366.

(73) Asada, M.; Furukawa, K.; Kantor, C.; Gahmberg, C. G.; Kobata, A. Structural Study of the Sugar Chains of Human Leukocyte Cell Adhesion Molecules CD11/CD18. *Biochemistry* **1991**, *30* (6), 1561–1571. https://doi.org/10.1021/bi00220a017.

(74) Miller, L. J.; Springer, T. A. Biosynthesis and Glycosylation of P150,95 and Related Leukocyte Adhesion Proteins. *J. Immunol.* **1987**, *139* (3), 842–847. https://doi.org/10.4049/jimmunol.139.3.842.

(75) Bellis, S. L. Variant Glycosylation: An Underappreciated Regulatory Mechanism for Β1 Integrins. *Biochim. Biophys. Acta BBA-Biomembr.* **2004**, *1663* (1–2), 52–60.

(76) Qian, S.; Golubnitschaja, O.; Zhan, X. Chronic Inflammation: Key Player and Biomarker-Set to Predict and Prevent Cancer Development and Progression Based on Individualized Patient Profiles. *EPMA J.* **2019**, *10* (4), 365–381. https://doi.org/10.1007/s13167-019-00194-x.

(77) Pshezhetsky, A. V.; Ashmarina, L. I. Desialylation of Surface Receptors as a New Dimension in Cell Signaling. *Biochem. Mosc.* **2013**, *78* (7), 736–745. https://doi.org/10.1134/S0006297913070067.

(78) Jia, F.; Howlader, M. A.; Cairo, C. W. Integrin-Mediated Cell Migration Is Blocked by Inhibitors of Human Neuraminidase. *Biochim. Biophys. Acta - Mol. Cell Biol. Lipids* **2016**, *1861* (9), 1170–1179. https://doi.org/10.1016/j.bbalip.2016.06.013.

(79) Hata, K.; Koseki, K.; Yamaguchi, K.; Moriya, S.; Suzuki, Y.; Yingsakmongkon, S.; Hirai, G.; Sodeoka, M.; Von Itzstein, M.; Miyagi, T. Limited Inhibitory Effects of Oseltamivir and Zanamivir on Human Sialidases. *Antimicrob. Agents Chemother.* **2008**, *52* (10), 3484–3491. https://doi.org/10.1128/AAC.00344-08.

(80) Crucian, B.; Nelman-Gonzalez, M.; Sams, C. Rapid Flow Cytometry Method for Quantitation of LFA-1-Adhesive T Cells. *Clin. Vaccine Immunol.* **2006**, *13* (3), 403–408. https://doi.org/10.1128/CVI.13.3.403-408.2006.

(81) Cherry, L. K.; Weber, K. S. C.; Klickstein, L. B. A Dominant Jurkat T Cell Mutation That Inhibits LFA-1-Mediated Cell Adhesion Is Associated with Increased Cell Growth1. *J. Immunol.* **2001**, *167* (11), 6171–6179. https://doi.org/10.4049/jimmunol.167.11.6171.

(82) Zhang, Y.; Albohy, A.; Zou, Y.; Smutova, V.; Pshezhetsky, A. V.; Cairo, C. W. Identification of Selective Inhibitors for Human Neuraminidase Isoenzymes Using C4,C7-Modified 2-Deoxy-2,3-Didehydro-N-Acetylneuraminic Acid (DANA) Analogues. *J. Med. Chem.* **2013**, *56* (7), 2948–2958. https://doi.org/10.1021/jm301892f.
(83) Richards, M. R.; Guo, T.; Hunter, C. D.; Cairo, C. W. Molecular Dynamics Simulations of Viral Neuraminidase Inhibitors with the Human Neuraminidase Enzymes: Insights into Isoenzyme Selectivity. *Bioorg. Med. Chem.* **2018**, *26* (19), 5349–5358. https://doi.org/10.1016/j.bmc.2018.05.035.
(84) Ribeiro, J. P.; Pau, W.; Pifferi, C.; Renaudet, O.; Varrot, A.; Mahal, L. K.; Imberty, A. Characterization of a High-Affinity Sialic Acid-Specific CBM40 from Clostridium Perfringens and Engineering of a Divalent Form. *Biochem. J.* **2016**, *473* (14), 2109–2118. https://doi.org/10.1042/BCJ20160340.
(85) Bojar, D.; Meche, L.; Meng, G.; Eng, W.; Smith, D. F.; Cummings, R. D.; Mahal, L. K. A Useful Guide to Lectin Binding: Machine-Learning Directed Annotation of 57 Unique Lectin Specificities. *ACS Chem. Biol.* **2022**, *17* (11), 2993–3012. https://doi.org/10.1021/acschembio.1c00689.
(86) Wang, Y. Sialidases From Clostridium Perfringens and Their Inhibitors. *Front. Cell. Infect. Microbiol.* **2020**, *9*. https://doi.org/10.3389/fcimb.2019.00462.
(87) Ju, T.; Cummings, R. D. A Unique Molecular Chaperone Cosmc Required for Activity of the Mammalian Core 1 Beta3-Galactosyltransferase. *Proceeding Natl. Acad. Sci.* **2002**, *99* (26), 16613–16618. https://doi.org/10.1073/pnas.262438199.
(88) Kreisel, W.; Volk, B. A.; Büchsel, R.; Reutter, W. Different Half-Lives of the Carbohydrate and Protein Moieties of a 110,000-Dalton Glycoprotein Isolated from Plasma Membranes of Rat Liver. *Proc. Natl. Acad. Sci.* **1980**, *77* (4), 1828–1831. https://doi.org/10.1073/pnas.77.4.1828.
(89) Tauber, R.; Park, C. S.; Reutter, W. Intramolecular Heterogeneity of Degradation in Plasma Membrane Glycoproteins: Evidence for a General Characteristic. *Proc. Natl. Acad. Sci.* **1983**, *80* (13), 4026–4029. https://doi.org/10.1073/pnas.80.13.4026.
(90) Hunter, C. D.; Cairo, C. W. Detection Strategies for Sialic Acid and Sialoglycoconjugates. *ChemBioChem* **2024**, e202400402. https://doi.org/10.1002/cbic.202400402.
(91) Woska, J. R.; Last-Barney, K.; Rothlein, R.; Kroe, R. R.; Reilly, P. L.; Jeanfavre, D. D.; Mainolfi, E. A.; Kelly, T. A.; Caviness, G. O.; Fogal, S. E.; Panzenbeck, M. J.; Kishimoto, T. K.; Giblin, P. A. Small Molecule LFA-1 Antagonists Compete with an Anti-LFA-1 Monoclonal Antibody for Binding to the CD11a I Domain: Development of a Flow-Cytometry-Based Receptor Occupancy Assay. *J. Immunol. Methods* **2003**, *277* (1), 101–115. https://doi.org/10.1016/S0022-1759(03)00176-5.
(92) Woska, J. R., Jr.; Shih, D.; Taqueti, V. R.; Hogg, N.; Kelly, T. A.; Kishimoto, T. K. A Small-Molecule Antagonist of LFA-1 Blocks a Conformational Change Important for LFA-1 Function. *J. Leukoc. Biol.* **2001**, *70* (2), 329–334. https://doi.org/10.1189/jlb.70.2.329.
(93) Steffan, J. J.; Snider, J. L.; Skalli, O.; Welbourne, T.; Cardelli, J. A. Na+/H+ Exchangers and RhoA Regulate Acidic Extracellular pH-Induced Lysosome

Trafficking in Prostate Cancer Cells. *Traffic* **2009**, *10* (6), 737–753. https://doi.org/10.1111/j.1600-0854.2009.00904.x.
(94) MacDonald, E.; Forrester, A.; Valades-Cruz, C. A.; Madsen, T. D.; Hetmanski, J. H. R.; Dransart, E.; Ng, Y.; Godbole, R.; Shp, A. A.; Leconte, L.; Chambon, V.; Ghosh, D.; Pinet, A.; Bhatia, D.; Lombard, B.; Loew, D.; Larsen, M. R.; Leffler, H.; Lefeber, D. J.; Clausen, H.; Blangy, A.; Caswell, P.; Shafaq-Zadah, M.; Mayor, S.; Weigert, R.; Wunder, C.; Johannes, L. Growth Factor-Triggered de-Sialylation Controls Glycolipid-Lectin-Driven Endocytosis. *Nat. Cell Biol.* **2025**. https://doi.org/10.1038/s41556-025-01616-x.
(95) Weichert, A.; Faber, S.; Jansen, H. W.; Scholz, W.; Lang, H. J. Synthesis of the Highly Selective Na+/H+ Exchange Inhibitors Cariporide Mesilate and (3-Methanesulfonyl-4-Piperidino-Benzoyl) Guanidine Methanesulfonate. *Arzneimittelforschung.* **1997**, *47* (11), 1204–1207.
(96) Aits, S.; Kricker, J.; Liu, B.; Ellegaard, A.-M.; Hämälistö, S.; Tvingsholm, S.; Corcelle-Termeau, E.; Høgh, S.; Farkas, T.; Holm Jonassen, A.; Gromova, I.; Mortensen, M.; Jäättelä, M. Sensitive Detection of Lysosomal Membrane Permeabilization by Lysosomal Galectin Puncta Assay. *Autophagy* **2015**, *11* (8), 1408–1424. https://doi.org/10.1080/15548627.2015.1063871.
(97) Zou, Y.; Albohy, A.; Sandbhor, M.; Cairo, C. W. Inhibition of Human Neuraminidase 3 (NEU3) by C9-Triazole Derivatives of 2,3-Didehydro-N-Acetyl-Neuraminic Acid. *Bioorg. Med. Chem. Lett.* **2010**, *20* (24), 7529–7533. https://doi.org/10.1016/j.bmcl.2010.09.111.
(98) Pinho, S. S.; Reis, C. A. Glycosylation in Cancer: Mechanisms and Clinical Implications. *Nat. Rev. Cancer* **2015**, *15* (9), 540–555. https://doi.org/10.1038/nrc3982.
(99) *Cancer Stem Cell Markers and Related Network Pathways*; Tanabe, S., Ed.; Advances in Experimental Medicine and Biology; Springer International Publishing: Cham, 2022; Vol. 1393. https://doi.org/10.1007/978-3-031-12974-2.
(100) Pearce, O. M. T.; Läubli, H. Sialic Acids in Cancer Biology and Immunity. *Glycobiology* **2016**, *26* (2), 111–128. https://doi.org/10.1093/glycob/cwv097.
(101) *Essentials of Glycobiology*, 4th ed.; Varki, A., Cummings, R. D., Esko, J. D., Stanley, P., Hart, G. W., Aebi, M., Mohnen, D., Kinoshita, T., Packer, N. H., Prestegard, J. H., Schnaar, R. L., Seeberger, P. H., Eds.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor (NY), 2022.
(102) Baumann, A.-M. T.; Bakkers, M. J.; Buettner, F. F.; Hartmann, M.; Grove, M.; Langereis, M. A.; Groot, R. J. de; Mühlenhoff, M. 9-O-Acetylation of Sialic Acids Is Catalysed by CASD1 via a Covalent Acetyl-Enzyme Intermediate. *Nat. Commun.* **2015**. https://doi.org/10.1038/ncomms8673.
(103) Kakugawa, Y.; Wada, T.; Yamaguchi, K.; Yamanami, H.; Ouchi, K.; Sato, I.; Miyagi, T. Up-Regulation of Plasma Membrane-Associated Ganglioside Sialidase (Neu3) in Human Colon Cancer and Its Involvement in Apoptosis Suppression. *Proc. Natl. Acad. Sci.* **2002**, *99* (16), 10718–10723. https://doi.org/10.1073/pnas.152597199.
(104) Ueno, S.; Saito, S.; Wada, T.; Yamaguchi, K.; Satoh, M.; Arai, Y.; Miyagi, T. Plasma Membrane-Associated Sialidase Is Up-Regulated in Renal Cell Carcinoma and

Promotes Interleukin-6-Induced Apoptosis Suppression and Cell Motility. *J. Biol. Chem.* **2006**, *281* (12), 7756–7764. https://doi.org/10.1074/jbc.M509668200.
(105) Kawamura, S.; Sato, I.; Wada, T.; Yamaguchi, K.; Li, Y.; Li, D.; Zhao, X.; Ueno, S.; Aoki, H.; Tochigi, T.; Kuwahara, M.; Kitamura, T.; Takahashi, K.; Moriya, S.; Miyagi, T. Plasma Membrane-Associated Sialidase (NEU3) Regulates Progression of Prostate Cancer to Androgen-Independent Growth through Modulation of Androgen Receptor Signaling. *Cell Death Differ.* **2012**, *19* (1), 170–179. https://doi.org/10.1038/cdd.2011.83.
(106) Uemura, T.; Shiozaki, K.; Yamaguchi, K.; Miyazaki, S.; Satomi, S.; Kato, K.; Sakuraba, H.; Miyagi, T. Contribution of Sialidase NEU1 to Suppression of Metastasis of Human Colon Cancer Cells through Desialylation of Integrin B4. *Oncogene* **2009**, *28* (9), 1218–1229. https://doi.org/10.1038/onc.2008.471.
(107) Yamanami, H.; Shiozaki, K.; Wada, T.; Yamaguchi, K.; Uemura, T.; Kakugawa, Y.; Hujiya, T.; Miyagi, T. Down-regulation of Sialidase NEU4 May Contribute to Invasive Properties of Human Colon Cancers. *Cancer Sci.* **2007**, *98* (3), 299–307. https://doi.org/10.1111/j.1349-7006.2007.00403.x.
(108) Gabr, S.; Sherif, Y.; Ghfar, A.; Alghadir, A. *Biomarkers in Disease: Methods, Discoveries and Applications*; 2017. https://doi.org/10.1007/978-94-007-7675-3.
(109) Zhang, Z.; Wuhrer, M.; Holst, S. Serum Sialylation Changes in Cancer. *Glycoconj. J.* **2018**, *35* (2), 139–160. https://doi.org/10.1007/s10719-018-9820-0.
(110) Shamberger, R. J. Serum Sialic Acid in Normals and in Cancer Patients. *J. Clin. Chem. Clin. Biochem.* **1984**, *22* (10), 647–651.
(111) Westman, J. S.; Yang, W. H.; Marth, J. D. Investigating the Functions of Endogenous Neuraminidases Neu1 and Neu3 in Blood Cell and Protein Homeostasis. *FASEB J.* **2020**, *34* (S1), 1–1.
(112) Yang, W.; Aziz, P. V.; Heithoff, D. M.; Mahan, M. J.; Smith, J. W.; Marth, J. D. An Intrinsic Mechanism of Secreted Protein Aging and Turnover. *Proc. Natl. Acad. Sci.* **2015**. https://doi.org/10.1073/pnas.1515464112.
(113) Warner, T. G.; O'Brien, J. S. Synthesis of 2'-(4-Methylumbelliferyl)-.Alpha.-D-N-Acetylneuraminic Acid and Detection of Skin Fibroblast Neuraminidase in Normal Humans and in Sialidosis. *Biochemistry* **1979**, *18* (13), 2783–2787. https://doi.org/10.1021/bi00580a014.
(114) Wang, Y. Sialidases From Clostridium Perfringens and Their Inhibitors. *Front. Cell. Infect. Microbiol.* **2020**, *9*, 462. https://doi.org/10.3389/fcimb.2019.00462.
(115) Ribeiro, J. P.; Pau, W.; Pifferi, C.; Renaudet, O.; Varrot, A.; Mahal, L. K.; Imberty, A. Characterization of a High-Affinity Sialic Acid-Specific CBM40 from Clostridium Perfringens and Engineering of a Divalent Form. *Biochem. J.* **2016**, *473* (14), 2109–2118. https://doi.org/10.1042/BCJ20160340.
(116) Pan, X.; De Britto Pará De Aragão, C.; Velasco-Martin, J. P.; Priestman, D. A.; Wu, H. Y.; Takahashi, K.; Yamaguchi, K.; Sturiale, L.; Garozzo, D.; Platt, F. M.; Lamarche-Vane, N.; Morales, C. R.; Miyagi, T.; Pshezhetsky, A. V. Neuraminidases 3 and 4 Regulate Neuronal Function by Catabolizing Brain Gangliosides. *FASEB J.* **2017**, *31* (8), 3467–3483. https://doi.org/10.1096/fj.201601299R.