SIALIDASE (NEU1) PROMOTES INTERLEUKIN-6

OVEREXPRESSION OF SIALIDASE (NEU1) PROMOTES INTERLEUKIN-6 INDUCED INFLAMMATION IN HUMAN NEUROGLIA AND MONOCYTIC THP-1 CELLS

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ABSTRACT

Mammalian sialidases are hydrolytic enzymes that initiate the removal of terminal α2-3, α2-6 and α2-8 sialic acid residues from various sialylated glycoconjugates. Sialidases are reportedly involved in numerous cellular functions involving proliferation, differentiation, antigenic expression, inflammation and the tumorigenicity of malignant cells. Recently, sialidase has been implicated in various immune signaling pathways, involving immune effector cells, such as activated lymphocytes and macrophages. The human lysosomal sialidase gene encodes a 46 kD glycoprotein which exists in a multienzyme complex with β-galactosidase and PPCA. Neurodegenerative diseases such as Tay-Sachs and Sandhoff are characterized by the progressive storage of glycoproteins and sialylated oligosaccharides in the nervous system. The induction of inflammatory mediators is a critical step in the pathogenesis of neurodegeneration that remains largely undefined. As such, an *in vitro* model of Tay-Sachs disease was used to identify potential mediators involved in disease progression. In addition, we have used the THP-1 monocytic cell line as a model of human macrophages which play a key role in potentiating a variety of immune responses.

Translocation of neu1 from lysosomes to the cell surface and the resulting interaction with signaling molecules suggests neu1 is involved in the regulation of immune activities. We have investigated the role of sialidase on CD44 expression, an inflammation-associated glycoprotein found on the cell surface. Our data indicate that sialidase interacts with CD44 on the cell surface which may contribute to disease progression in Tay-Sachs disease. We illustrate that overexpression of sialidase

stimulates interleukin-6 (IL-6) secretion in both human Tay-Sachs neuroglia and THP-1 derived macrophages. Moreover, the sialidase inhibitor 2-deoxy-2, 3-dehydro-N-acetyl-neuraminic acid (DANA) was found to attenuate IL-6 secretion and sialidase expression in THP-1 derived macrophages.

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At last, I would like to thank my beloved Arvie for his infinite love and devotion; I feel truly blessed to be at your side.

This work is dedicated to my mom and dad for their endless love and encouragement – Thank you.

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LIST OF ABBREVIATIONS

AP2 Adaptor Protein 2

AP3 Adaptor Protein 3

AD Alzheimer's Disease

cAMP Cyclic Adenosine Monophosphate

CT-1 Cardiotrophin

CLC Cardiotrophin-like Cytokine

CNTF Ciliary Neurotrophic Factor

CD Cluster of Differentiation

CNS Central Nervous System

DANA 2-deoxy-2, 3-dehydro-N-acetyl-neuraminic acid

DMEM Dulbecco's Modified Eagle's Medium

ELISA Enzyme-Linked Immunosorbent Assay

FBS Fetal Bovine Serum

FITC Fluoro-Isothiocyanate

GAG Glycosaminoglycan

GFAP Glial Fibrillary Acidic Protein

gp130 Glycoprotein 130

HA Hyaluronan

Ig Immuno Globuline

IL-1 beta Interleukin-1 beta

IL-4 Interleukin-4

IL-6 Interleukin-6

IL-8 Interleukin-8

IL-11 Interleukin-11

INF-gamma Interferon gamma

JAK Janus Kinase

JNK c-Jun N-terminal Kinase

LIF Leukemia Inhibitory Factor

LPS Lipopolysaccharide

MALII *Maackia Amurensis* II

MAPK Mitogen Activated Protein Kinase

MHC Major Histocompatibility Complex

MHC II Major Histocompatibility Complex II

MOI Mode of Infection

MS Multiple Sclerosis

MU-NANA 4-methylumbelliferyl-α-N-acetyl-D-neuraminic acid

Neu5Ac N-acetylneuraminic acid

NCAM Neural cell adhesion molecules

NO Nitric Oxide

OSM Oncostatin M

PD Parkinson's Disease

PBS Phosphate Buffered Saline

PCR Polymerase Chain Reaction

PGF Prostaglandin F

PKA Protein Kinase A

PKC Protein Kinase C

PLP1 Proteolipid Protein-1

PMA Phorbol 12-myrsitate 13-acetate

PNA *Arachis hypogaea*

PPCA Protective Protein/Cathepsin A

RPMI Roswell Park Memorial Institute

SNA *Sambucus nigra*

SD Sandhoff disease

STAT Signal Transducer and Activator of Transcription

STAT2 Signal Transducer and Activator of Transcription 2

TSD Tay-Sachs disease

TLR Toll-like Receptor

TNF-alpha Tumor Necrosis Factor alpha

VCAM Vascular Cell Adhesion Molecule-1

WGA Wheat Germ Agglutinin

CHAPTER 1

Introduction

INTRODUCTION

Glycobiologists study the structure and biochemistry of saccharides, also known as glycans, and their structural relationships and functionality in biological systems. Glycans are ubiquitous and found on all eukaryotic cell surfaces, they naturally occur on proteins and lipids, yielding glycoproteins and glycolipids. Glycans can form a wide variety of structures on a protein or lipid backbone by the addition or removal of glycans via the actions of glycosyltransferases and glycosidases (Dube and Bertozzi, 2005). These structures change with their surrounding cellular conditions and yet, the simplest modification can mediate molecular interactions, thus altering the structure and thereby function of these molecules. For example, the (de)glycosylation of proteins and lipids can effect protein-carbohydrate interactions, and subsequently, mediate energy metabolism and cell signaling (Varki et al., 1999). Further insight into the glycosylation status, structural and biological interactions of glycoconjugates has the potential to elucidate complex oligosaccharide structures which potentially code biological information. Notably, hundreds of genes regulating N-glycan biosynthesis (glycotransferase and glycosidase) are highly conserved and are found in all eurkaryotic cells (Varki et al., 1999). Determining the structural complexity of glycans by modifying specific glycan residues via glycotransferases and glycosidase will help elucidate the physiology and function of naturally occurring glycans.

In general, there are two major classes of oligosaccharides that attach to proteins; N-linked and O-linked glycans. The O-linked glycans are attached to serine (Ser) and

threonine (Thr) residues; whereas N-linked glycans are equally specific, occurring on asparagine (Asn) residues (Varki et al., 1999). Typically, glycan structures can vary, forming linear glycans to more common branched structures, including the N- and O-linked complex subtypes (Dube and Bertozzi, 2005 and Varki et al., 1999). The biological processes associated with extracellular N-glycan linkages in vertebrates vary and to date, include hematopoiesis, immune function, inflammation responses and tumor metastasis (Varki et al., 1999). Because the vast array of glycan structures and their differences in expression level, stability or function, the data remains staggering. However, slow and calculated measures are starting to pay dividends, elucidating the molecular mechanisms governing glycan structure and function.

SIALIC ACID AND LECTIN BINDING

Sialic acids or neuraminic acids represent a super family of unique 9-carbon sugars which are widely distributed in viruses, bacteria, fungi, avian and mammalian species (Monti et al., 2002 and Varki et al., 1999). To date, nearly 40 structurally distinct forms of sialic acid have been observed and the most common form of sialic acid found in humans exists as N-acetylneuraminic acid (Neu5Ac) (Varki et al., 1999). Because of their abundant nature, structural versatility and terminal position, sialic acid residues play an important role in determining structural modifications on oligosaccharides (Keppler et al., 1999 and Varki et al., 1999). The structural diversity among sialic acid residues differ by altering α- linkages involving, the most common α-2,3 α-2,6 and α-2,8 linkages (Varki et al., 1999). Because glycoproteins and glycolipids possess a variety of oligosaccharides on the cell surface, different terminal residues and linkages are easily

identified with the help of specific antibodies and glycan binding plant lectins (Varki et al., 1999). Any modifications in chemical structure of sialic acid can help to elucidate the structural complexity of glycans which in turn, mediate vital cellular responses. Altering carbohydrate structures and exploiting the specific binding capacity of lectins in the protein-carbohydrate recognition system provides a powerful tool in elucidating the structural complexity of glycans. Sialylated glycans attached to terminal galactose via an α-2,6 linkage and to a lesser extent, via an α-2,3 linkage are preferentially recognized by *Sambucus nigra* agglutinin (SNA) and weakly recognized by wheat germ agglutinin (WGA) (Varki et al., 1999). Peanut agglutinin (PNA) from *Arachis hypogaea* preferentially recognizes non-sialylated glycans, specifically a commonly occurring oligosaccharide structure, galactose which is present in membrane associated glycoproteins, glycolipids and gangliosides (Perrin et al, 1997).

SIALIDASES

Sialidases are hydrolytic enzymes that initiate the removal of terminal α2-3, α2-6 and α2-8 sialic acid residues from a number of sialylated glycoconjugates, while leaving the glycan chain attached to the lipid or protein (deGeest et al., 2002, Keppler 1999 and Monti et al., 2002). Sialidases are reportedly involved in numerous cellular functions involving proliferation, differentiation, antigenic expression and inflammation (Monti et al., 2002 and Varki 1997). In addition, differential cell surface sialylation is implicated in metastastic behaviour of malignant cells (Keppler et al., 1999). Removal of cell surface sialic acids may unmask and potentially alter recognition ability and may regulate immune cell interactions. To date, several mammalian sialidases have been cloned and

they are classified into three distinct types: lysosomal (Neu1), cytosolic (Neu2) and plasma membrane (Neu3) (Igdoura et al 1998 and Monti et al., 2002). A recent report has yielded a novel mammalian sialidase (Neu4) localized in the mitochondria (Yamaguchi et al., 2005). Several biochemical properties vary among sialidases, in addition to cellular localization and substrate specificity (Carrillo et al., 1997 and Igdoura et al., 1998). However, molecular cloning and characterization of sialidases have revealed a high degree of homology between Neu4 and both Neu2 and Neu3 sialidases (Monti et al., 2004). Human sialidases are of particular interest because defective or deficient lysosomal sialidase is clearly linked with two neurodegenerative storage disorders: sialidosis and galactosialidosis, whereby glycolipid intermediates accumulate in the lysosome (Bonten et al., 1996, Monti et al., 2002, and Seyrantepe et al., 2003).

LYSOSOMAL SIALIDASE (NEU1)

The human lysosomal sialidase gene encodes a 44 kD and 46kD glycoprotein that exists as part of a multienzyme complex containing β-galactosidase (β-gal), protective protein or cathepsin A (PPCA) and N-acetyl-galactosamine-6-sulfate sulfatase (d'Azzo et al., 1982, van der Spoel et al., 1998 and Pshezhetsky and Ashmarina, 2001). Any structural mutation leading to a decrease in lysosomal sialidase produces the lysosomal storage disease sialidosis; whereas a deficiency in sialidase, along with deficient PPCA has the potential to cause the disorder galactosialidosis (deGeest et al., 2002 and Seyrantepe et al., 2003). Previous studies have found that the functional activity of Neu1 requires association with both β-galactosidase and cathepsin A (van der Spoel et al., 1998 and Pshezhetsky and Ashmarina, 2001). The lysosomal sialidase (Neu1) gene spans 3.7

kb of DNA and has been mapped to the human histocompatibility locus on chromosome 6p21 (Pshezetshy et al., 1997 and Monti et al., 2002). The primary structure has revealed homology among mammalian species, sharing conserved amino acid residues, including a F(R)YIP motif located at the N-terminus and a series of repeated aspartic boxes featured throughout (Roggentin et al., 1989 and Monti et al., 2002). These conserved sequences and shared chemical structure are illustrated by various species, suggesting a common phylogenetic origin (Crennell et al., 1993). In the murine counterpart, the Neu1 gene was mapped to the histocompatibility complex on chromosome 17 (Womack et al., 1981). The sequence analysis of both human and mouse cDNA show greater than 80% homology between these two species (Carrillo et al., 1997 and Pshezhetsky et al., 1997).

CELLULAR TRANSLOCATION OF NEU1

Immunoelectron microscopy demonstrated the intracellular distribution of Neu1 along the lysosomal membrane and lumen, in addition to the plasma membrane (Lukong et al., 2001 and Seyrantepe et al., 2003). The complex regulation of Neu1 trafficking involves synthesis in the endoplasmic reticulum, followed by targeting to the lysosomes, (van der Spoel et al., 1998). Lysosomal sialidase is then translocated toward the cell surface where it is retained on the plasma membrane (Lukong et al., 2001). The association of adaptor complexes, adaptor protein 2 (AP2) and adaptor protein (AP3) is required for formation of endosomal and lysosomal vesicles which are required for proper intracellular transport (Lukong et al., 2001). The specific mechanisms for intracellular sorting of Neu1 to the lysosomes remains unknown, however the formation with PPCA remains critical for complex association and activation (van der Spoel et al.,

1998). A recent study has shown the translocation of lysosomal sialidase to the plasma membrane following differentiation of human blood monocytes (Liang et al., 2006). Neu1 is involved in lysosomal catabolism and has been recently reported to translocate from lysosomes to the cell surface, whereby inducing human blood T-cells via the T-cell receptor, thus suggesting a role in immune function (Wang et al., 2004). These targeting studies are important in explaining just how lysosomal sialidase interacts at the plasma membrane with various surface receptors and possibly other signaling mediators. Furthermore, these reports reveal lysosomal sialidase as a potent mediator of cellular activation during an immune response and, thus provide insight into the physiological processing of this multi-functional enzyme.

NEURODEGENERATIVE DISEASES: TAY-SACHS AND SANDHOFF

Tay-Sachs and Sandhoff diseases (TSD and SD) are inherited lysosomal storage disorders characterized by the accumulation of G_{M2} gangliosides (Myerowitz et al., 2002 and Phaneuf et al., 1996). G_{M2} accumulation is associated with an inflammatory response that results in progressive neurodegeneration, central nervous system (CNS) dysfunction, and programmed cell death in neurons (Wada et al., 2000). Neuronal apoptosis and proinflammatory mediators are also features of more common neurodegenerative disorders, such as Alzheimer's disease (AD) and Parkinson's disease (PD) (Venters et al., 1999). TSD and SD are characterized by the absence of lysosomal β-hexosaminidase (Hex A) and the inability to catabolize G_{M2} gangliosides and related glycolipids (Igdoura et al., 1999, Phaneuf et al., 1996 and Huang et al., 1997). Gangliosides are sialic acid containing glycosphingolipids (GSL) which are found on the surface of most cell

membranes and exist predominantly in the mammalian nervous system (Phaneuf et al., 1996). Several different forms of gangliosides are found in the brain, all sharing one common feature; they are composed of a lipid anchor, ceramide. Ceramide is attached to an externally oriented glycan chain, with variable numbers of sialic acid residues (Pyo et al., 1999). In brain gangliosides, these glycan chains are sequentially synthesized through a biosynthetic pathway mainly involving glycosyltransferases. Alternatively, the chains are sequentially broken down via a catabolic pathway mainly involving hydrolases within lysosomes.

The products of 3 genes are required for normal catabolism of G_{M2} gangliosides. Defects in the α-subunit causes TSD (the α-chain deficiency) and defects in the β-subunit can cause SD (β-chain deficiency). In addition, defects in G_{M2} activator causes variant forms of G_{M2} gangliosidosis (Phaneuf et al., 1996). Any one of these deficiencies will result in massive accumulation of G_{M2} and glycolipids. In turn, this accumulation initiates an inflammatory-like response causing severe neurodegeneration and dysfunction throughout the nervous system (Werth et al., 2001). Currently, there is no treatment for G_{M2} gangliosidosis and in the most severe clinical cases, death occurs between 2-5 years of age (Jeyakumar et al., 2003 and Wada et al., 2000). While the pathology in TSD and SD is similar in humans, the mouse models of these two diseases show extraordinary differences. There is evidence for sialidase mediated G_{M2} catabolism; the murine models of TSD show mice with minor neurological pathology and minimal G_{M2} accumulation, suggesting the existence of an alternative pathway for degradation of G_{M2} (Monti et al., 2002). Tay-Sachs mice ($hexa^{-/-}$) exploit a sialidase-

mediated metabolic bypass of the genetic defect, whereas Sandhoff mice ($hexb^{-/-}$) are symptomatic and experience neurodegeneration in the CNS (Sango et al., 1995 and Phaneuf et al., 1996). It was discovered that unlike humans, this efficient conversion of G_{M2} to the corresponding asialo derivatives, glycolipids G_{A2} by lysosomal sialidase provides an alternative pathway for ganglioside degradation in TSD (Sango et al., 1995 and Phaneuf et al., 1996).

INFLAMMATION-MEDIATED NEURODEGENERATION

Microglial cells are the brain resident macrophages which perform the immune surveillance in the CNS (Pyo et al., 1999). Naturally quiescent, microglia will assume an active state following infection or injury, rapidly responding to subtle changes in the surrounding microenvironment (Duke et al., 2004). Resting microglial cells can also become activated during chronic neurodegenerative disorders such as Parkinson's disease, Alzheimer's disease and multiple sclerosis (Gonzalez-Scarano and Baltuch, 1999 and Kim and Joh, 2006). In lysosomal storage diseases, this activation occurs when enzyme-deficient cells are unable to catabolize gangliosides, resulting in an accumulation of glycolipid intermediates which is followed by the migration of activated microglial cells to the site of injury (Gonzalez-Scarano and Baltuch, 1999 and Duke et al., 1999). Molecular targets of microglia activation in lysosomal storage disorders remain elusive, partly because microglia perform a dual role; they have a neuroprotective role while guarding the CNS, as well as a neurodegenerative role while intensifying neuronal inflammation (Gonzalez-Scarano and Baltuch, 1999). As a primary response, activated microglia respond to trauma or the presence of pathogens by migrating to the site of

injury where they may proliferate, however, this activity is potentially deleterious. According to Wada and colleagues, the inflammatory response is mediated by microglia, whereby neuronal storage leads to neuronal damage, which ultimately leads to microglial activation and expansion (Wada et al., 2000). This expansion initiates neuronal apoptosis, causing additional damage through excessive glycolipid storage to an already compromised CNS. The inflammatory process in the CNS is believed to play an important role in the signaling pathway through which dysfunction of the CNS ensues, causing neuronal cell death (Wada et al., 2000). In Tay-Sachs and Sandhoff diseases, the accumulation of gangliosides initiates microglial activation, triggering the signaling of various proinflammatory cytokines (Jeyakumar et al., 2003). An inflammatory reaction involving the secretion of TNF-α and IL-6 by activated microglia thus implicates these particular cytokines as important mediators in neurodegenerative disease which can trigger an apoptotic signaling cascade (Nagai et al., 2005 and Lucas et al., 2006).

Like other tissue macrophages during an inflammatory response, activated microglia can release chemokines, nitric oxide (NO) and cytokines such as TNF-α, IL-6 and interleukin-1β (IL-1β) which have the potential to amplify an immune response (Gonzalez-Scarano and Baltuch, 1999, Pyo et al., 1999, and Kim and Joh, 2006). Specifically, elevated levels of TNF-α, IL-1β and IL-6 have the potential to exacerbate pathogenesis of various inflammatory, autoimmune and neurodegenerative diseases (Kishimoto et al., 1995 and Van Wagoner and Benveniste, 1999). IL-6 is a ubiquitous cytokine which elicits both proinflammatory and anti-inflammatory effects in the CNS and is found to regulate numerous cellular functions in immunological responses (Kopf et

al., 1994 and Van Wagoner et al., 1999). The proinflammatory role of IL-6 is emphasized following infection or trauma and neurodegeneration, when expression of IL-6 is upregulated in the affected regions (Raivich et al., 1999). Moreover, an increase of IL-6 is widely considered an early indicator of tissue damage and is implicated in T cell activation and proliferation (Takai et al., 1988). IL-6 regulation is influenced by several proinflammatory agents and neurotransmitters leading to a dynamic role in inflammation (Raivich et al., 1999). *In vitro* experiments using human adult astrocytes indicate increasing IL-6 gene transcription in the presence of exogenous TNF-α and IL-1β; a 6.7-fold increase with TNF-α versus 13.3–fold increase with IL-1β (Van Wagoner et al., 1999). Therefore, in brain pathology, circulating cytokines such as TNF-α and IL-1β are essential mediators and inducers of chemokines and adhesion molecules, such as the TNF-α-induced expression of vascular cell adhesion molecule-1 (VCAM-1) (Van Wagoner et al., 1999 and Heinrich et al., 2003). The increased expression levels of proinflammatory cytokines TNF-α and IL-1β, following microglial activation and the recruitment of immune cells to the CNS may elicit the pathogenesis associated with CNS inflammation.

Cytokines are multifunctional and often exhibit redundancy in biological responses and these overlapping functions are attributed to the use of a common signal transduction receptor (Van Wagoner and Benveniste, 1999). In particular, IL-6 and associated molecules utilize a single receptor complex containing glycoprotein 130 (gp130) (Kishimoto et al., 1995). The family of IL-6 related cytokines using the membrane-bound gp130 complex includes IL-11, ciliary neurotrophic factor (CNTF),

leukemia inhibitory factor (LIF), oncostatin M (OSM), cardiotrophin (CT-1) and cardiotrophin-like cytokine (CLC) (Heinrich et al., 2003). Evidence suggests that activation of IL-6 is dependent upon association with the gp130 receptor complex, and although gp130 is ubiquitously expressed, receptor function is closely regulated (Heinrich et al., 2003). The interaction of IL-6 in the presence of IL-6 receptor (IL-6R) is essential for IL-6 expression, as it restricts the association of the gp130 receptor complex and ubiquitous IL-6 (Van Wagoner and Benveniste, 1999). IL-6 expression will activate the signal transducer and activator of transcription (STAT) signaling pathway via protein kinase C (PKC) and p38 mitogen-activated protein kinase (MAPK) (Chio et al., 2004). Therefore, it is compelling that IL-6 signal transduction modulates STAT signaling, a pathway previously associated with ganglioside-stimulated inflammation in activated microglia.

Apoptosis and an inflammatory response are observed in several neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, Tay-Sachs and Sandhoff disease (Hirsch et al., 1998, McGeer and McGeer, 1995, and Myerowitz et al., 2002). The role of inflammation-mediated neurodegeneration in G_{M2} gangliosidoses patients is compelling, but no causal role in humans has been established. Dissecting the inflammatory mediated response will help us uncover mechanisms of disease progression and novel molecular targets that may reveal potential targets for therapeutic opportunities. Our objectives are to examine the relationship between sialidase expression and its role in glial activation and to explore the mechanisms of inflammation associated with ganglioside accumulations. We propose these studies will increase our

knowledge of Tay-Sachs and Sandhoff diseases which can provide a basis for therapeutic interventions.

CHAPTER 2

Overexpression of sialidase (neu1) promotes interleukin-6 induced inflammation in Tay-Sachs neuroglia

ABSTRACT

The induction of inflammatory mediators in the pathogenesis of neurodegeneration remains largely undefined. A rare metabolic storage disorder, known as Tay-Sachs disease has provided evidence of a sialidase-mediated G_{M2} catabolism in the murine model; whereby the mice have shown enhanced activity toward ganglioside accumulations. As such, an *in vitro* model of Tay-Sachs disease was used to identify potential mediators involved in disease progression. Previous reports have suggested a possible interaction between sialidase and the inflammation-associated adhesion molecule CD44, which is found on the cell surface. The removal of sialic acids from the CD44 glycoprotein was found to activate cell migration and induce an immune response. Our present studies have characterized changes in glycosylation of CD44 in Tay-Sachs neuroglia, implicating the importance of glycosylation changes in inflammation-associated diseases.

Here we show increased CD44 expression levels in Tay-Sachs neuroglia when compared to normal cells. In addition, we illustrate a direct correlation between the overexpression of endogenous sialidase (neu1) and an increase in CD44 expression in normal and Tay-Sachs neuroglia. Furthermore, our data show that overexpression of sialidase is a potent inducer of IL-6 secretion, but not TNF-α. Although the role of CD44 in IL-6 secretion remains undetermined, these observations demonstrate CD44 as one of the relevant molecules affected by sialidase, which may contribute to disease progression in Tay-Sachs disease.

INTRODUCTION

Sialic acids are abundant in nature and widely distributed in viruses, bacteria, fungi, avian and mammalian species (Monti et al., 2002 and Varki et al., 1999). Because of their abundance, structural versatility and terminal position, sialic acid residues play an important role in determining structural and cellular modifications (Keppler et al., 1999 and Varki et al., 1999). Glycoproteins possess a variety of oligosaccharides on the cell surface and these terminal residues are easily identified with the help of specific antibodies and glycan binding plant lectins (Varki et al., 1999). The modifications in chemical structure of sialic acids help to elucidate the structural complexity of glycans which in turn, provide insight to vital cellular responses. Altering the cell surface landscape and exploiting the specific binding capacity of lectins provides a powerful tool in elucidating various molecular interactions.

Sialidases are hydrolytic enzymes that initiate the removal of terminal sialic acid residues from various substrates, including glycoproteins, gangliosides and oligosaccharides, while leaving the glycan chain attached to the lipid or protein (deGeest et al., 2002, Keppler 1999 and Monti et al., 2002). In mammals, sialidases are involved in cellular proliferation and differentiation, cell-cell interactions, antigen masking and tumorigenicity of malignant cells (Keppler 1999, Monti et al., 2002 and Varki 1997). The human lysosomal sialidase (Neu1) gene spans 3.7 kb and has been mapped to the human histocompatibility locus on chromosome 6p21 (Pshezetshy et al., 1997). The lysosomal sialidase gene (Neu1) encodes a 44–46 kD glycoprotein that exists in a multienzyme complex with β-galactosidase (β-gal) and protective protein/cathepsin A

(PPCA) (Bonten et al., 1995). Human sialidases are of particular interest because lysosomal sialidase provides an alternative pathway for ganglioside degradation in Tay-Sachs disease (Myerowitz et al., 2002). The human pathology in Tay-Sachs and Sandhoff diseases are similar and result in G_{M2} ganglioside accumulations and neurodegeneration in the CNS; whereas the murine models show extraordinary differences. The murine model of Tay-Sachs show mice with minor neurological pathology and minimal G_{M2} accumulation, suggesting the existence of an alternative degradative pathway involving sialidase mediated G_{M2} catabolism (Sango et al., 1995 and Phaneuf et al., 1996). Although, the role of inflammation-mediated neurodegeneration in G_{M2} gangliosidoses patients is compelling, no causal role in humans has been established.

Neu1 was reportedly involved in cell signaling, where it has been shown to translocate from lysosomes to the cell surface, whereby inducing human blood T-cells via the T-cell receptor, suggesting a role in immune function (Wang et al., 2004). Interestingly, sialidase activity has been shown to directly desialylate the inflammation-associated adhesion molecule CD44, which is found on the cell surface (Gee et al., 2003 and Katoh et al., 1999). The removal of sialic acids from the cell surface of glycoprotein CD44 was found to activate cell migration and induce an immune response (Katoh et al., 1999 and Skelton et al., 1998). At the cell surface, interaction with CD44 is proposed to present select cytokines to neighboring cells, thereby amplifying the potent cytokine, TNF-α (Webb et al., 1990). Moreover, the report indicated that secretion of TNF-α was observed within 3 to 6 hours upon induction with CD44, thus corresponding to early events of T cell activation (Webb et al., 1990).

CD44 glycoprotein is highly sialylated, belonging to a family of surface molecules known as cell adhesion molecules (CAMs) (Johnson et al., 2000). CD44 is a widely distributed and highly polymorphic glycoprotein involved in various cellular interactions including cell adhesion and migration, lymphocyte activation, immune function, haematopoiesis and tumor metastasis (Bourguignon et al., 1997, Johnson et al., 2000 and Vivers et al., 2002). A high degree of amino acid sequence homology exists among the cell adhesion molecules and high conservation of N-linked glycosylation sites were discovered across species (Nottenburg et al., 1989 and Pure and Cuff, 2001). Ranging in molecular weight from 80-250 kDa, numerous CD44 isoforms are produced by post-translational modifications including glycosylation at the extracellular domain (Gallatin et al., 1991 and Ponta et al., 2003). Since oligosaccharides are required for cellular recognition and interaction, modifying cell surface sialylation may potentially expose alternative targets, ultimately affecting binding of inflammatory mediators and extracellular components. Because CD44 is heavily glycosylated on the cell surface (Figure 1) and easily remodeled, perhaps stimulating effects of other accessory molecules on the cell surface may help to identify a novel molecular target in the inflammatory mediated response leading to disease progression. Here, we show that overexpression of endogenous sialidase (neu1) can alter specific glycans on CD44 in human neuroglia. Furthermore, overexpression of sialidase has induced IL-6 secretion, but not TNF-α, suggesting participation of sialylation during an immune response. However, the physiologic triggering of IL-6 via CD44 remains unclear.

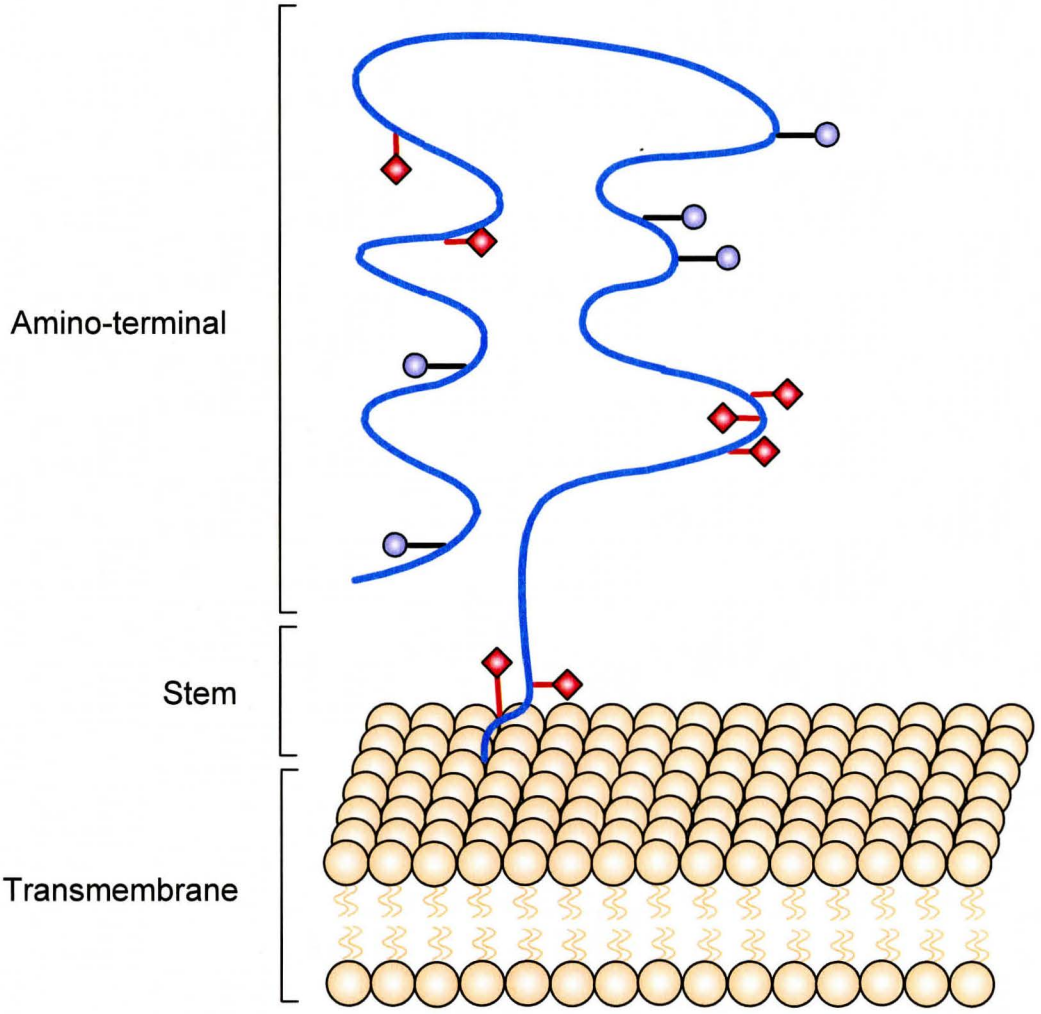

Figure 1. Glycosylation sites on CD44. There are 5 N-glycosylation sites (in purple) and 7 O-glycosylation sites (in red) which are found throughout the amino-terminal domain and stem of CD44.

Adapted from Ponta et al., 2003

MATERIALS AND METHODS

Cell Culture and Reagents

Primary neuroglia cultures were derived from the cerebella of aborted Tay-Sachs and normal fetuses, kindly provided by Steve Brooks at the Brooklyn Jewish Medical Centre, New York. Tay-Sachs primary cell line synthesizes G_{M2} gangliosides which accumulate in lysosomes (Hoffman et al., 1977). In this study, we utilize an *in vitro* model of Tay-Sachs disease to observe the effects of sialidase overexpression on CD44 in neuroglia cells from normal and Tay-Sachs patients. Neuroglia were cultured in high-glucose (4.5 mg/L) Dulbecco's modified Eagle's medium (DMEM) (Hyclone) supplemented with 10-15% fetal bovine serum (FBS), supplemented with 50 units/ml penicillin, 50 μg/ml streptomycin, 500 μg amphotericin B and 4 mM L-glutamine (Invitrogen) and maintained at 37°C and 5% atmospheric CO_2. Cells were counted prior to infection with differing concentrations of adenovirus overexpressing sialidase (Ad-Sial), using the improved Neubauer haemocytomer and plated at 1.0 X 10^6 per 60 mm dish (Corning).

Cytokine Measurement

Neuroglia were washed 2X with 1X Phosphate Buffered Saline (PBS) and fresh media was replenished, in the presence or absence of LPS (*Escherichia coli* 0111:B4) purchased from Sigma-Aldrich (St. Louis, MO). During LPS treatment, the culture medium was changed to DMEM supplemented with low endotoxin FBS (Invitrogen). Cell culture medium was collected from triplicate samples following 18-20 hour

incubation with the endotoxin at differing concentration (100-10000 ng/ml). Media samples were analyzed for human interleukin-6 (IL-6), interleukin-4 (IL-4) and tumour necrosis factor-alpha (TNF-α) using the BD OptEIA™ Human ELISA Kit II, for instructions see manufacturer website (BD Biosciences). The minimum detectable limit of supernatant concentrations TNF-α was determined to be 2 pg/ml and 4.9 pg/ml for IL-6. The colorimetric reaction was read in a 96-well microplate reader at absorbance 450 nm using Safire microplate reader (TECAN).

Biotin-Avidin Lectin Binding

Since lectins recognize glycan structure independent of the polypeptide backbone, we were able to determine the glycosylation changes on specific molecular components. Neuroglia were collected and suspended in 1 ml lysis buffer (1% NP-40, 2mM phenylmethylsulphonyl fluoride and 0.1% deoxycholate) with a protease inhibitor cocktail (Roche), incubated overnight with 10-20 μg biotinylated lectins: *Maackia Amurensis II* (MALII), *Sambucus Nigra* (SNA) or *Peanut Agglutinin* (PNA) (Vector Laboratories). CD44 was precipitated through its glycans from lysates using streptavidin beads (Amersham Biosciences). Following overnight incubation at 4°C, cell lysates were centrifuged at 13,000 r.p.m. for 5 minutes; 1 ml of ice cold 1X PBS (Hyclone) was added to the pellet to wash, vortexed and centrifuged again. Finally, the pellet was reduced in 2X SDS-PAGE sample buffer containing β-mercaptoethanol and boiled for 5 minutes at 100 °C. Samples were separated by SDS-PAGE and transferred to Biotrace nitrocellulose membrane (PALL) and blocked in 20-25 ml of 5% non-fat milk in 1X TBST. The membranes were then incubated with anti-CD44 (Santa Cruz), rabbit anti-

sialidase (S. A. Igdoura Lab) and anti-β-actin (ICN) overnight in 5% non-fat milk in 1X TBST. Blots were incubated with horseradish peroxidase-labeled secondary antibodies in 1X TBST for 1 hour at room temperature. Proteins were then visualized using ECL western blotting detection kit (Amersham Biosciences) and exposed to Hyperfilm (Amersham Biosciences). Protein concentrations were determined using BioRad Protein Assay and microplate reader at absorbance 595 nm (TECAN).

RESULTS

Using a Novel Human neuroglia cell population

In a previous study, a comparative microarray analysis between the normal and Tay-Sachs neuroglia was performed in our laboratory using GE Healthcare Bioarrays. The previous study revealed 2 novel primary neuroglial cell lines composed of different cell types which include early neuronal and glial markers, astrocytes and oligodendrocytes. From the data, we found early oligodendrocyte markers, such as proteolipid protein-1 (PLP1) and claudin-11 being expressed, as well as the early astrocyte marker, vimentin. Interestingly, the neuroglia population does not exhibit classical behaviour typical of known glial population described previously (Raivich et al., 1999). For example, these cells do not respond when activated with varying concentrations of lipopolysaccharide (LPS) which is found on the outer membrane of gram negative bacteria. Since the Toll-like receptor (TLR) family was found to recognize LPS, this response can be attributed to very low mRNA levels of TLR-1 and TLR-4 in the neuroglia. Such interesting characteristics provide evidence for an

embryonic origin for these 2 cell lines (normal and Tay-Sachs cells) and support their utilization as a model to study the neuroinflammatory mechanisms underlying progression of Tay-Sachs disease.

CD44 gene and protein expression by neuroglia isolated from normal and Tay-Sachs patients

When we examined our bioarray data and compared CD44 gene expression between normal and Tay-Sachs, we found CD44 was upregulated 1.7-fold in Tay-Sachs neuroglia. This increase was confirmed at the protein level whereby measuring the band densitometry in CD44 expression between normal and Tay-Sachs cells, we have observed a 1.25-fold increase in CD44 expression in Tay-Sachs neuroglia (Figure 2).

Modulation by desialylation and N-glycosylation: Impact of glycosylation changes on CD44 in neuroglia between normal and Tay-Sachs patients

In order to examine the impact of sialidase overexpression on the sialylation status of CD44, we infected normal and Tay-Sachs neuroglia with adenovirus expressing sialidase. We then lysed the cells and pulled down α2,6 sialylated conjugates using the lectin SNA and followed it by western blot analysis for CD44. Surprisingly, our results indicate that in normal and Tay-Sachs cells the amount of sialylated CD44 was significantly increased after sialidase overexpression (Figure 3). An increase in sialylation following overexpression of sialidase could be explained through the action of sialyltransferases which are responsible for the addition of terminal sialic acids. To examine if sialidase overexpression would increase the amount galactose-conjugated

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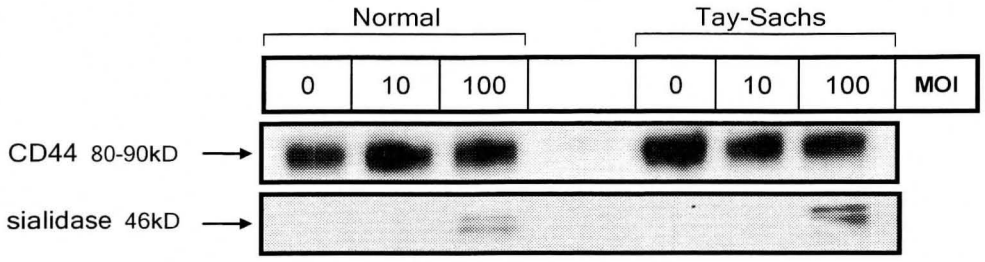

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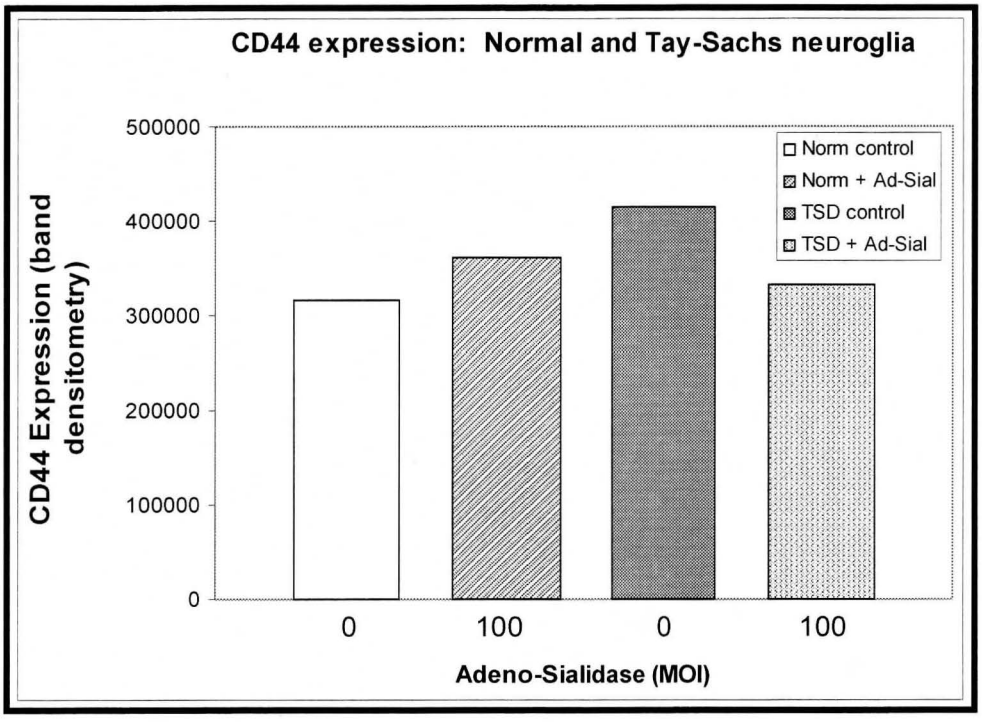
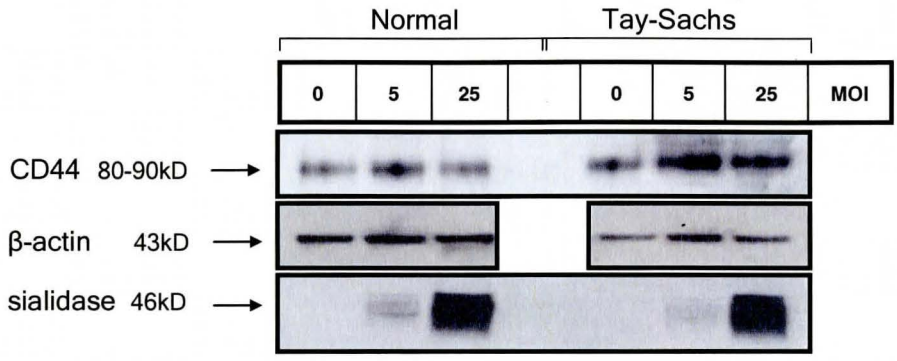


Figure 2. A) CD44 western blot. Normal and TSD neuroglia were infected with Ad-Sialidase [0, 10 and 100 MOI] and incubated for 68 hours. Samples were normalized for protein and run on 7% resolving gel, transferred to nitrocellulose membranes and incubated overnight with CD44 antibody. When comparing the mock-infected samples, the western blot demonstrates increased expression of CD44 in Tay-Sachs neuroglia versus the Normal neuroglia. B) Band densities were quantified using densitometry and represent CD44 expression in normal and Tay-Sachs neuroglia. Data represent one experiment.

A)



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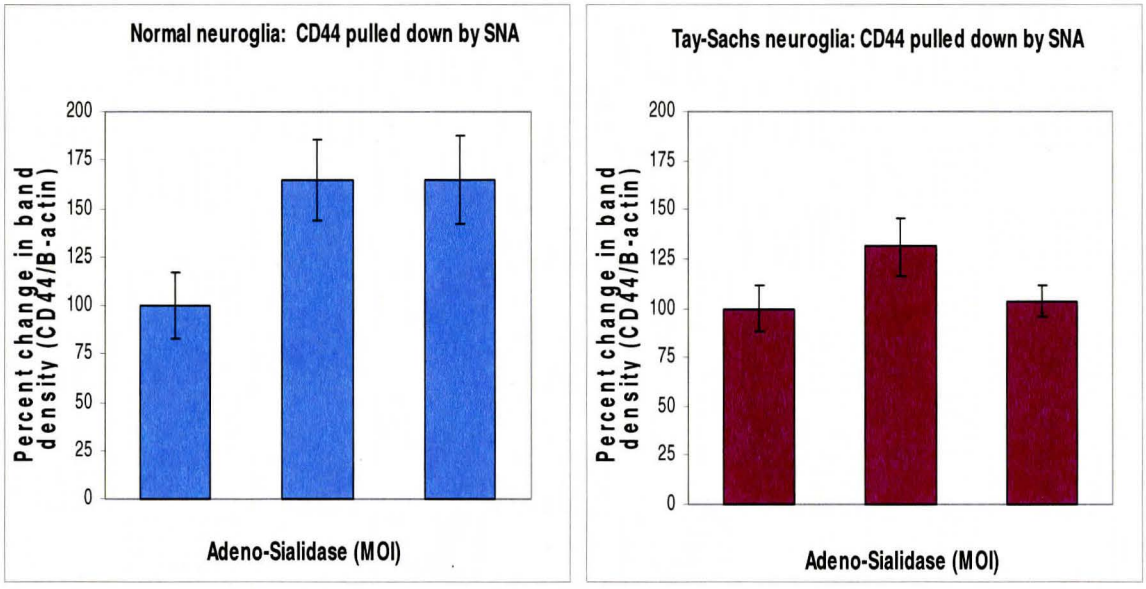
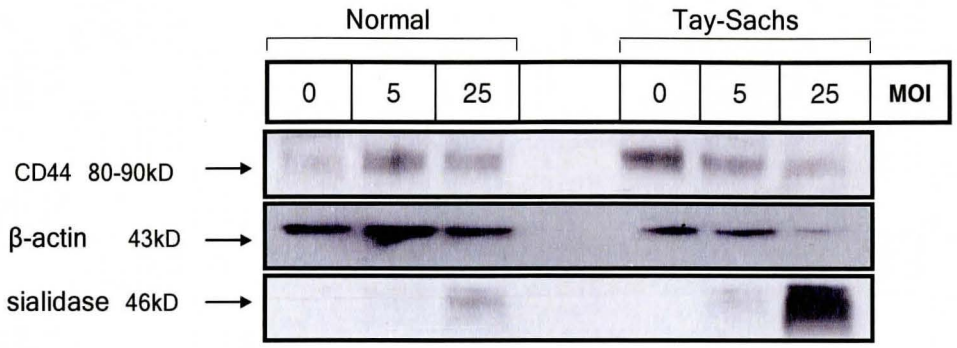


Figure 3. **A)** SNA pull down and CD44 western blot. Normal and TSD neuroglia were infected with Ad-Sialidase [0, 5, 25 MOI] and incubated for 68 hours. Samples were harvested, incubated with biotinylated SNA lectin and pulled down with avidin-sepharose and were run on 6% resolving gel, transferred to nitrocellulose membrane and incubated overnight with CD44 antibody. Cell lysates were blotted for β-actin and sialidase. **B)** Band densitometry represented as percent change of SNA pull down and CD44 western blot. Observe the 1.5-fold increase in CD44 expression in normal cells overexpressing sialidase; 1.25-fold increase in CD44 expression in Tay-Sachs cells overexpressing sialidase. Data shown represent the mean ± S.E. from 3 separate experiments.

CD44, we overexpressed sialidase in normal and Tay-Sachs neuroglia cells and pulled down galactose-β1,3-acetylgalactosamine conjugates using PNA lectin. The pull downs were analysed for the amount of CD44 by western blotting. Our results in normal neuroglia appear to follow our predictions with the levels of galactose-conjugated CD44 increasing by 5-fold. However, with the Tay-Sachs cells the trend was reversed with sialidase overexpression resulting in a significant reduction in galactose-bound CD44 (Figure 4). This supports that some desialylation is taking place in normal neuroglia cells overexpressing sialidase, but not in Tay-Sachs neuroglia overexpressing sialidase. This highlights the impact of β-hexosaminidase A deficiency (Tay-Sachs disease) on CD44 glycosylation and potentially its function. In another experiment, we infected normal and Tay-Sachs neuroglia cells with Ad-Sialidase to overexpress sialidase, lysed the cells and then pulled down α2,3-sialylated conjugates using the lectin MALII (Figure 5). The resulting pull down was probed for CD44. The results indicate that after sialidase overexpression, significantly higher levels of CD44 carrying α2,3-sialylation were detected.

In order to examine the possibility of CD44 upregulation following sialidase overexpression, we treated sialic-conjugates pulled down with SNA, as well as galactose-conjugates pulled down PNA with N-glycosidase F. This deglycosylation step was done to examine if the higher CD44 levels were in fact due to a response in sialidase overexpression. Our results show little impact of sialidase overexpression on the overall sialylation of CD44, illustrated by the absence of gel mobility shift following deglycosylation (Figures 6 and 7).

A)



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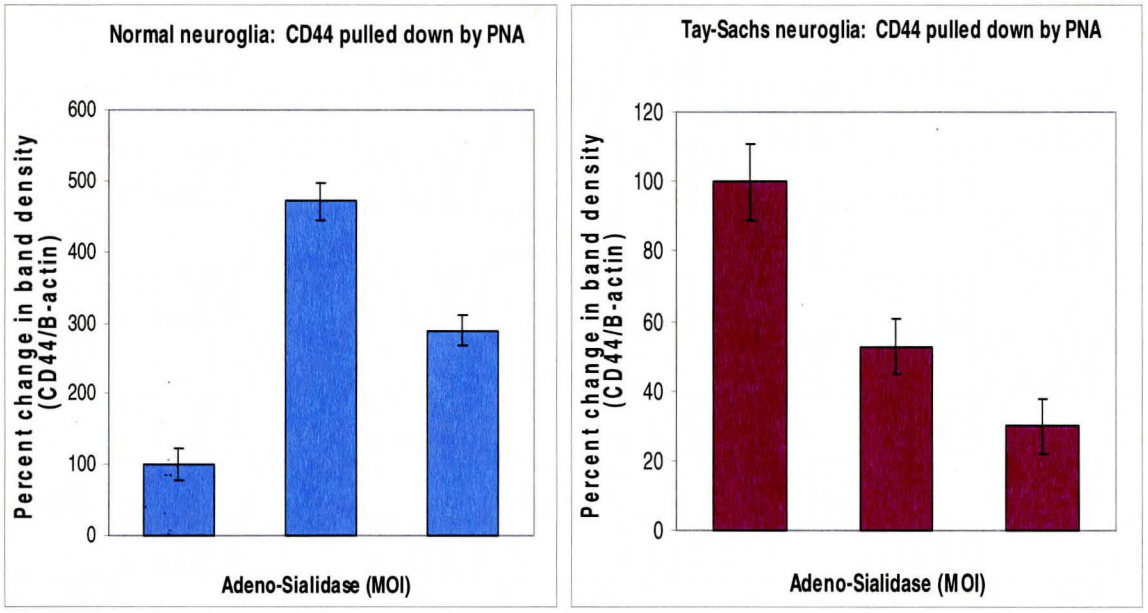
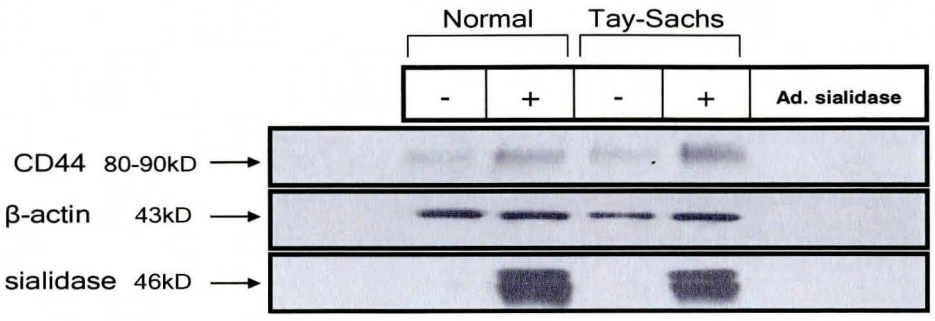


Figure 4. **A)** PNA pull down and CD44 western blot. Normal and TSD neuroglia were infected with Ad-Sialidase [0, 5, 25 MOI] and incubated for 68 hours. Samples were harvested and run on 6% resolving gel, transferred to nitrocellulose membranes and incubated overnight with CD44 antibody. Cell lysates were also blotted for β-actin and sialidase. **B)** Band densitometry represented as percent change of PNA pull down and CD44 western blot. Observe the 5-fold increase in CD44 expression in normal cells overexpressing sialidase. Alternatively, 1.5-fold reduction in CD44 expression in Tay-Sachs cells overexpressing sialidase. Data shown represent the mean ± S.E. from 2 separate experiments.

A)



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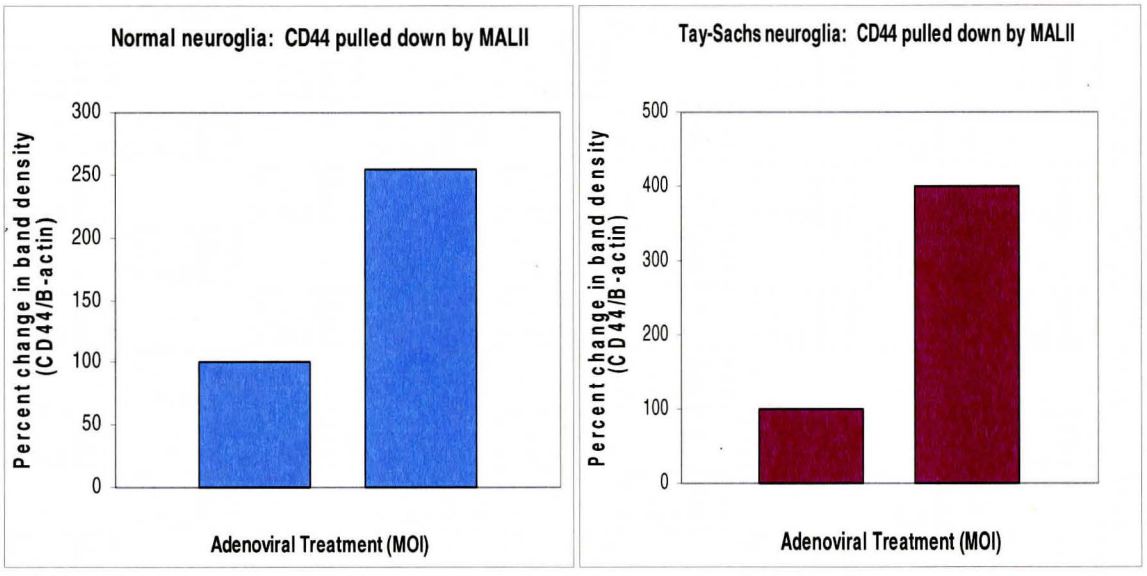

Figure 5. **A)** *Maackia Amurensis* II (MALII) pull down and CD44 western blot. Normal and TSD neuroglia were infected with Ad-Sial and further incubated for 68 hours. Samples were harvested, incubated with biotinylated lectin and pulled down with avidin-sepharose, then run on 6% resolving gel, transferred to nitrocellulose membrane and incubated overnight with CD44 antibody. Cell lysates were blotted for β-actin and sialidase **B)** Band densities were quantified using densitometry. Both normal and Tay-Sachs neuroglia overexpressing Ad-Sial show a 2.5-fold and 4-fold increase in CD44, respectively when compared to mock infected samples. Data are representative of one experiment.

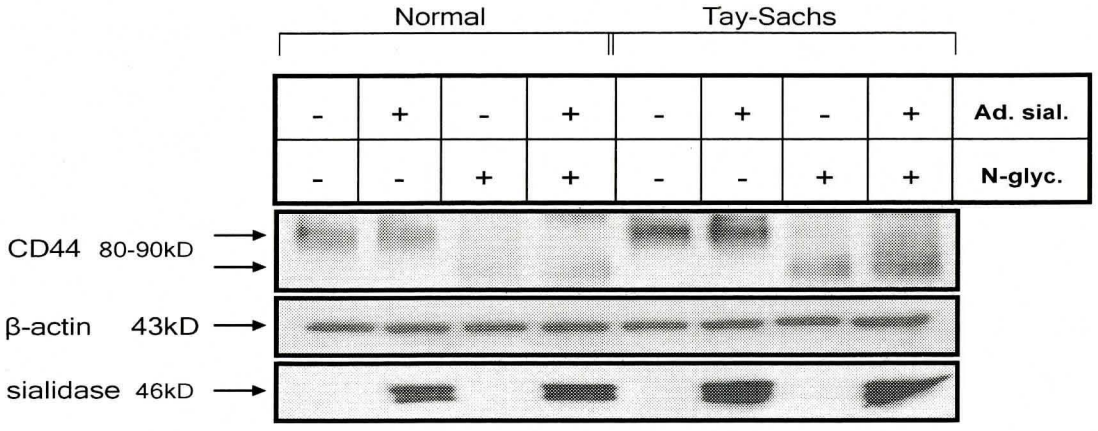

Figure 6. SNA pull down and CD44 western blot. Normal and TSD neuroglia were infected with Ad-Sial. for 30 minutes. Following infection, samples were incubated for a further 68 hours. Samples were harvested in 1 ml of solubilizing buffer each and the lysates incubated overnight with 20 μg of biotinylated SNA at 4° C and pulled down using streptavidin sepharose. The pellet was then washed 3X with 1X PBS and incubated overnight with N-glycosidase at 37°C. Following overnight treatment, run on 6% resolving gel, transferred to nitrocellulose membranes and incubated overnight with CD44 antibody. Band densities were quantified using densitometry. In normal and Tay-Sachs neuroglia, the amount of sialic-bound CD44 increased after sialidase treatment. A similar pattern is observed following overnight treatment with N-glycosidase.

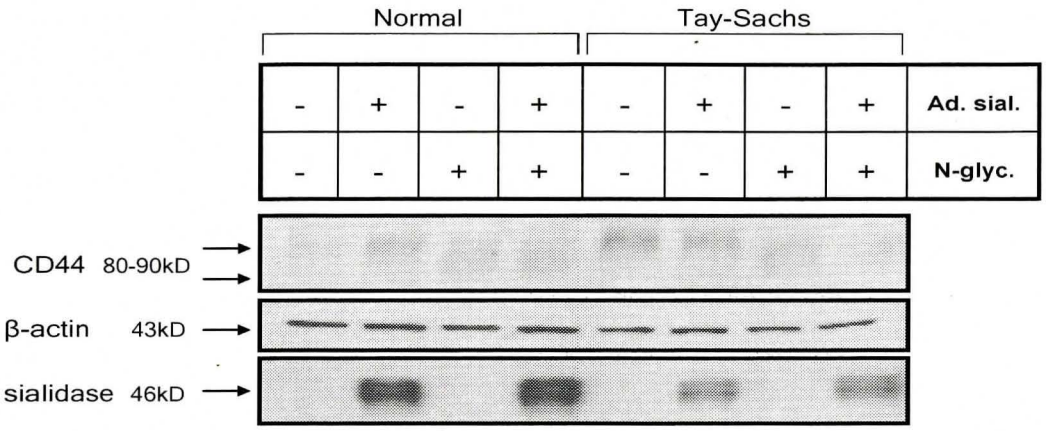

Figure 7. PNA pull down and CD44 western blot. Normal and TSD neuroglia were infected with Ad-Sial. for 30 minutes and incubated for a further 68 hours. Samples were harvested in 1 ml of solubilizing buffer and the lysates incubated overnight with 15 μg of biotinylated PNA at 4° C and pulled down using streptavidin sepharose. The pellet was then washed 3X with 1X PBS and incubated overnight with N-glycosidase at 37°C. Following incubation, samples were run on 6% resolving gel, transferred to nitrocellulose membranes and incubated overnight with CD44 antibody. Cell lysates were blotted for β-actin and sialidase. Band densities were quantified using densitometry. Normal neuroglia overexpressing sialidase show 3-fold increase in galactose-bound CD44 expression while galactose-bound CD44 was reduced in response to sialidase overexpression in Tay-Sachs neuroglia. Tay-Sachs cells treated with N-glycosidase show a 2-fold reduction in the amount of unglycosylated CD44.

Induction of IL-6, but not TNF-α by overexpression of sialidase in neuroglia from Tay-Sachs patients

In order to characterize lysosomal storage-mediated cytokine expression, we examined normal and Tay-Sachs neuroglia for endogenous cytokine secretion involving IL-6, IL-4 and TNF-α. To determine the change of IL-6 during an immune response, normal and Tay-Sachs neuroglia were stimulated overnight with differing concentrations (100-10000 ng/ml) of LPS and cytokine production was determined using enzyme linked immunoassays (ELISA). Initial results confirmed that stimulation with LPS resulted in a significant increase in IL-6 production in both normal and Tay-Sachs neuroglia, approximately 8X ($p<0.0007$) and 2X ($p<0.006$), respectively (Figure 8). Similarly, both neuroglia cell populations which were induced to overexpress sialidase showed a significant increase in IL-6 expression when compared to mock-infected cells (Figure 9 and 11). Our results show a direct effect of endogenous sialidase expression on IL-6 production in neuroglial cells (Figure 10). Furthermore, when Tay-Sachs neuroglia were infected with Ad-Sialidase in the presence of a sialidase inhibitor, 2-deoxy-2, 3-dehydro-N-acetyl-neuraminic acid (DANA), sialidase expression was greatly reduced. Future experiments will determine if the inhibitor DANA can diminish the stimulatory effect of sialidase overexpression on IL-6 secretion in these cells. Most surprisingly, IL-4 and TNF-α expression in both normal and Tay-Sachs neuroglia cells were at baseline levels, in the presence of *Eschericia coli* derived LPS (data not shown). In addition normal and Tay-Sachs neuroglia overexpressing sialidase did not produce detectable levels of TNF-α

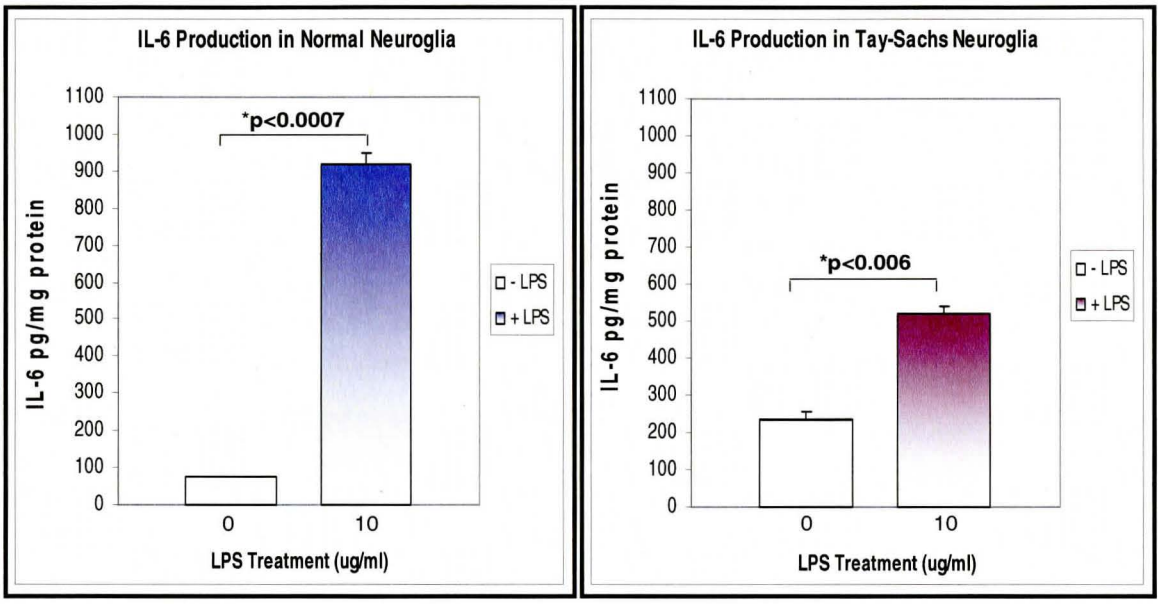

Figure 8. Measuring IL-6 secretion in response to lipopolysaccharide in Normal and Tay-Sachs neuroglia. Neuroglia were treated with 10 μg/ml of LPS or vehicle an incubated at 37 °C for 18 hours prior to human IL-6 ELISA assay. Treatment with LPS induced significant increases in IL-6 secretion in the normal ($p<0.0007$) and Tay-Sachs ($p<0.006$) cells. Protein concentration of cell lysates was measured using Bradford protein assay. Data shown represent the mean ± S.D. of triplicate samples and are representative of data from 3 separate experiments.

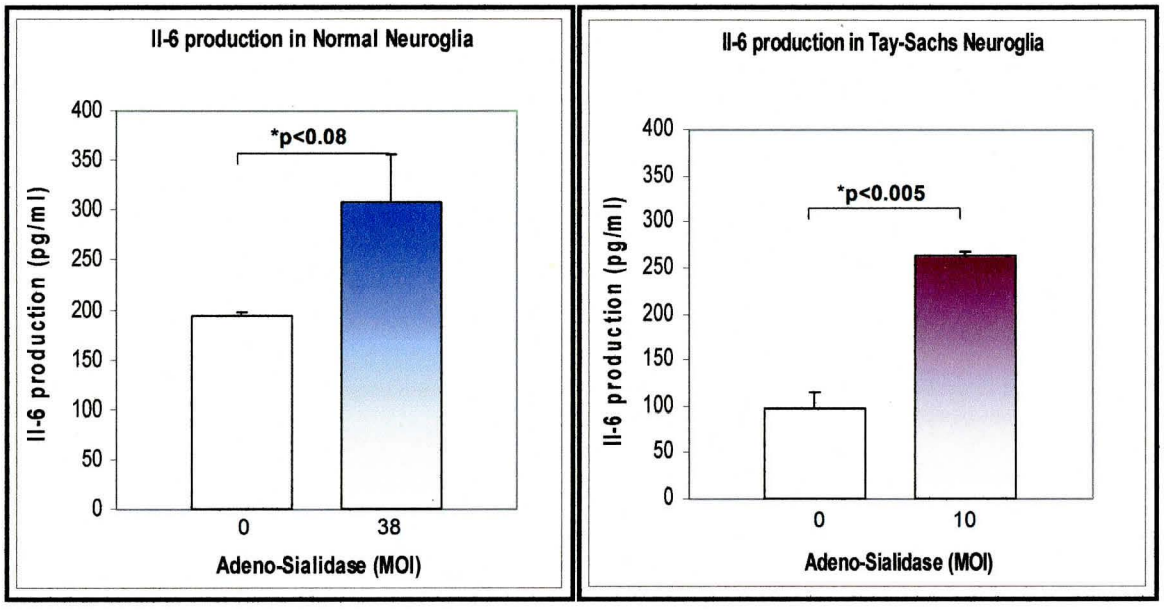

Figure 9. The effect of sialidase overexpression on IL-6 secretion in Normal and TSD neuroglia. Normal and Tay-Sachs neuroglia were infected with Ad-Sial or mock infected for 30 minutes and further incubated at 37 °C for 68 hours prior to human IL-6 ELISA. Overexpression of sialidase in both normal ($p<0.08$) and Tay-Sachs ($p<0.005$) neuroglia show an increase in IL-6 secretion. Data shown represent the mean ± S.D. of triplicate samples and are representative of data from 2 separate experiments.

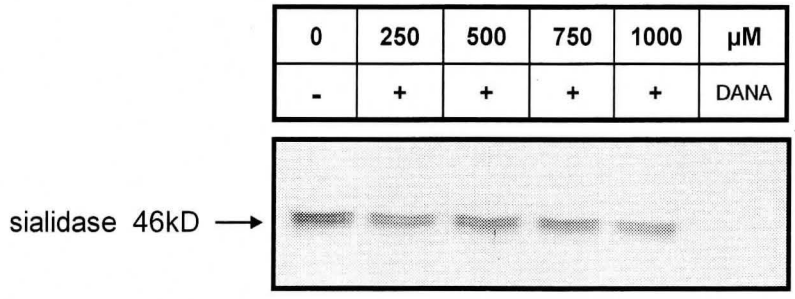

Figure 10. Sialidase inhibitor, DANA reduces sialidase expression in Tay-Sachs neuroglia. Tay-Sachs neuroglia were overexpressed with 10 MOI Ad-Sial. and treated with varying concentrations of sialidase inhibitor (µM) for 68 hours. Samples were harvested and run on 7% resolving gel, transferred to nitrocellulose membrane and incubated overnight with sialidase antibody. The above immunoblot shows a reduction in sialidase expression in Tay-Sachs samples treated with the sialidase inhibitor.

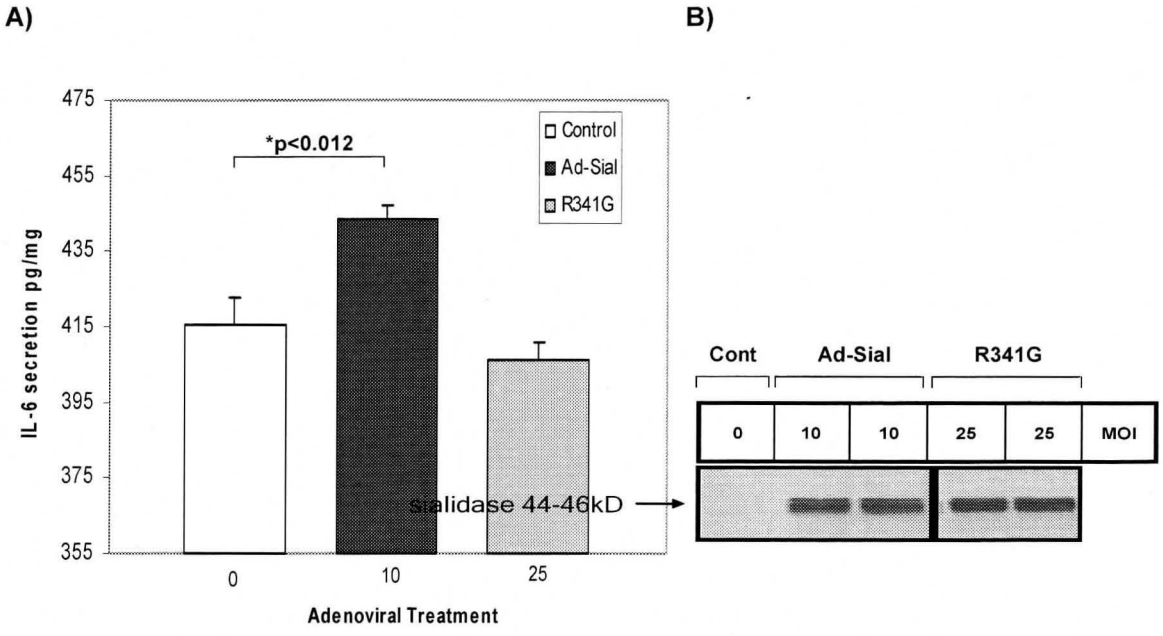

Figure 11. **A)** IL-6 activity in response to over expression of sialidase. Tay-Sachs neuroglia were treated with 10 MOI Ad-Sialidase and 25 MOI Ad. R341G (mutant sialidase) for 30 minutes and incubated for a further 68 hours prior to human IL-6 ELISA assay. IL-6 secretion from the mock-infected sample is equivalent to samples infected with catalytically inactive Ad. R341G ($p<0.012$). **B)** Sialidase immunoblot show Tay-Sachs samples infected with 10 MOI Ad-Sial. and 25 MOI Ad. R341G. Samples were resolved on 7% SDS-polyacrylamide gel, transferred to nitrocellulose membrane and incubated overnight with sialidase antibody.

cytokine (data not shown). These observations suggest an alternative signaling pathway employed by these cells for the induction of IL-6 secretion that is independent of the typical proinflammatory signaling pathway.

To confirm the effect is due to sialidase, we infected Tay-Sachs neuroglia cells with adenoviruses expressing either normal human sialidase (Ad-Sial) or catalytically inactive human sialidase (R431G mutation). The mutant sialidase was previously characterized in our laboratory and was shown to be targeted correctly, however catalytically inactive. Our results show that while overexpression of normal sialidase induced IL-6 secretion, the catalytically inactive sialidase had little on IL-6 (Figure 12). This result unequivocally demonstrates that IL-6 secretion in neuroglial cells was dependent on sialidase activity. Furthermore, using an *in vitro* model of Tay-Sachs disease, we show that by overexpressing mouse sialidase in human neuroglia we have observed a reduction in IL-6 secretion (Figure 12).

DISCUSSION

Lysosomal sialidases are of particular interest because they provide an alternative pathway for ganglioside degradation in Tay-Sachs disease (Myerowitz et al., 2002). The murine model of Tay-Sachs show mice with minor neurological pathology and minimal G_{M2} accumulation, suggesting the existence of an alternative degradative pathway involving sialidase mediated G_{M2} catabolism (Sango et al., 1995 and Phaneuf et al., 1996). In Tay-Sachs neuroglia overexpressing mouse sialidase, we observe a reduction in IL-6 secretion, which could explain how Tay-Sachs mice ($hexa^{-/-}$) show minor neuro-

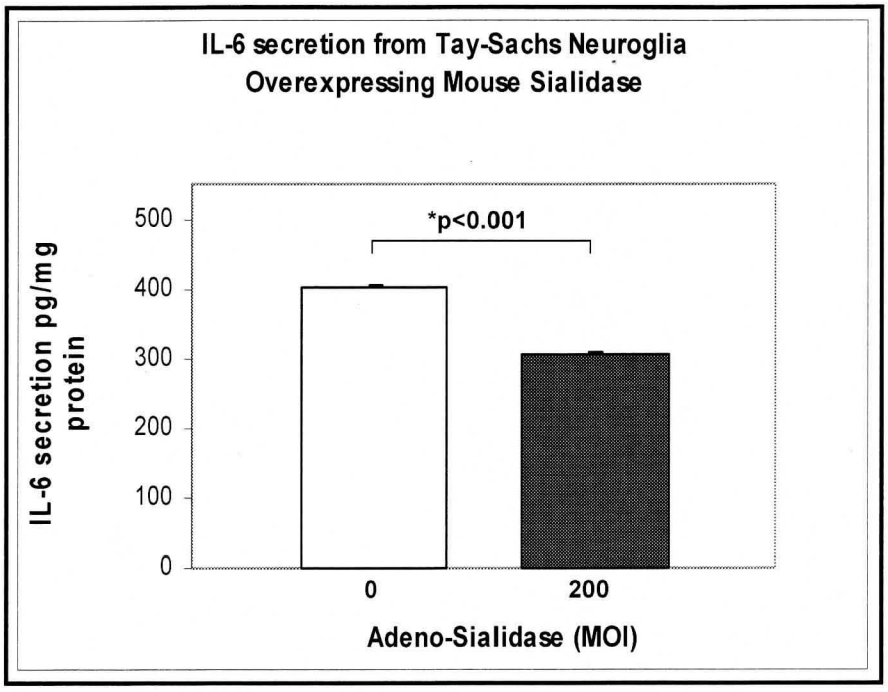

Figure 12. The effect of mouse sialidase overexpression on IL-6 secretion in Tay-Sachs neuroglia. Tay-Sachs neuroglia were infected with 200 MOI mouse sialidase or mock infected for 30 minutes and further incubated at 37 °C for 68 hours prior to human IL-6 ELISA. Overexpression of mouse sialidase in Tay-Sachs neuroglia show a significant reduction in IL-6 secretion; this result is opposite the significant increase observed in Tay-Sachs neuroglia infected with the human Ad-Sial. Data shown represent the mean ± S.D. of triplicate samples and are representative of data from 1 experiment.

logical pathology, thus implicating lysosomal sialidase in the regulation of the immune response.

Because cytokines are multifunctional and often exhibit redundancy in biological responses, determining function within dynamic relationships are often difficult to ascertain (Van Wagoner and Benveniste, 1999). IL-6 is a ubiquitous cytokine which elicits both proinflammatory and anti-inflammatory effects in the CNS and is found to regulate numerous cellular functions in immunological responses (Kopf et al., 1994 and Van Wagoner et al., 1999). As a central regulator of the immune response, IL-6 directly affects neurons and neuroglia, including oligodendrocytes and astrocytes (Raivich et al, 1999). Generally, IL-6 remains at low levels in the brain, however, IL-6 expression is upregulated in affected regions following infection or trauma and neurodegeneration (Raivich et al., 1999). Previously reviewed, increased IL-6 expression was produced by mice neuroglia cells due to injury in the CNS (Raivich et al., 1999). Moreover, IL-6 is widely considered an early indicator of tissue damage and is implicated in T cell activation and proliferation (Takai et al., 1988). IL-6 regulation is influenced by several proinflammatory agents and neurotransmitters leading to a dynamic role in inflammation (Raivich et al., 1999). *In vitro* experiments using human adult astrocytes showed a 6.7-fold increase in IL-6 gene transcription in the presence of exogenous TNF-α, suggesting TNF-α acts as an inducer of IL-6 associated inflammation in the CNS (Van Wagoner et al., 1999).

TNF-α levels are reported to be upregulated in neurodegenerative diseases such as multiple sclerosis and Alzheimer's disease, consistent with an involvement in

inflammatory processes (Venters et al., 1999). It is not surprising then to see reports of disease progression in Sandhoff mice associated with a 15X increase in TNF-α gene expression when compared to the control mice at 4 months of age (Wada et al., 2000). Furthermore, microglia cultured from 1-3 day old rats were treated with 50 μg/ml gangliosides for varying amounts of time induced 1000-fold increase in TNF-α release (Pyo et al., 1999). TNF-α induced microglial activation is exacerbated by the addition of gangliosides in already damaged brain cells (Pyo et al., 1999). In severe brain pathology, TNF-α is expressed by neurons and glial cells and is associated with an apoptotic wave found in regions where activated microglia were identified (Raivich et al., 1999).

During an inflammatory response, activated microglia and other tissue macrophages can secrete potentially lethal chemokines, nitric oxide and cytokines, thus amplifying an immune response (Gonzalez-Scarano and Baltuch, 1999, Pyo et al., 1999 and Kim and Joh, 2006). In the injured CNS, elevated levels of TNF-α and IL-6 appear to exacerbate the pathogenesis of various inflammatory and neurodegenerative diseases (Kishimoto et al., 1995 and Van Wagoner and Benveniste, 1999). The coordination of immune responses within the CNS ensues, including an increase in cell adhesion molecules and migration of inflammatory cells (Nagai et al., 2005).

Several physiological roles have been assigned to the cell surface protein CD44 involving cellular and cell-matrix adhesion through the interaction with hyaluronan (Nagai et al., 2005). Previous reports indicate immune reactive cells monocytes and leukocytes, initiating adhesion and cell migration and therefore, an immune response by remodeling cell surface CD44 (Katoh et al., 1999 and Nagai et al., 2005). Specifically,

reports have shown that treatment with N-glycosidase and sialidase increased CD44 binding affinity for HA, subsequently altering CD44 function in numerous cell types (Katoh et al., 1995 and Lesley et al., 1995). In addition, it has been suggested that inhibition of CD44 binding is caused by terminal sialic acids, attenuating HA recognition function (Katoh et al., 1995). The activation of N-glycosylation sites within CD44-HA binding domains was found to regulate CD44 function (English et al., 1998). The regulation of CD44 function by N-glycosylation of these CD44-HA binding domains were found to bear terminal α2,3 linkages of sialic acid, the removal of which led to increased binding of HA (English et al., 1998). These studies reveal exactly how glycan and linkage specificity can modify CD44 interactions and ultimately, CD44 function.

Circulating cytokines such as IL-6 and TNF-α are essential mediators and often inducers of chemokines and adhesion molecules, such as the TNF-α-induced expression of vascular cell adhesion molecule-1 (VCAM-1) (Van Wagoner et al., 1999 and Heinrich et al., 2003). Removal of cell surface sialic acids may unmask and potentially alter ligand recognition and may induce specific changes in the immune response. Desialylation reduces the amount of galactose-bound CD44 in Tay-Sachs neuroglia, implicating glycosidic changes as molecular mechanisms for structural changes on the cell surface. The increased expression levels of proinflammatory cytokines IL-6 and TNF-α following microglial activation and the recruitment of immune cells to the CNS may elicit the pathogenesis associated with CNS inflammation. Therefore, it is compelling that overexpression of sialidase increases production of IL-6 in Tay-Sachs neuroglia, a model of ganglioside-stimulated inflammation.

CHAPTER 3

Overexpression of sialidase (neu1) promotes interleukin-6 induced inflammation in human monocytic THP-1 cells

ABSTRACT

Recent reports have implicated the translocation of sialidase (neu1) to the cell membrane as a step during the activation of leukocytes. A distinct hallmark of immune cellular activation is the increased levels of proinflammatory cytokines. Here, we have used the THP-1 cell line as a model of human derived macrophages which become actively responsive to their extracellular environment and subsequently capable of mediating immune responses through the secretion of inflammatory mediators. We examined the modulatory effects of lipopolysaccharide (LPS) stimulation and overexpression of sialidase in the secretion of inflammatory cytokines tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) by THP-1 derived macrophages. We have observed that desialylation of human monocytes induced IL-6 secretion in differentiated THP-1 macrophages. In addition, we were able to diminish the impact of sialidase activity using a sialidase inhibitor, 2-deoxy-2,3-dehydro-N-acetylneuraminic acid (DANA) which consequently abolished IL-6 secretion and sialidase expression. Overall, we suggest participation of sialidase in the induction of proinflammatory cytokine secretion during an immune response.

INTRODUCTION

Much evidence has identified the increase of proinflammatory mediators as a characteristic feature of numerous brain pathologies including stroke, trauma, brain infections and chronic neurological disorders (Beutler and Cerami 1989, Akira and Kishimoto, 1992 and Vassalli, 1992). Neuronal apoptosis and proinflammatory mediators are common features of many neurodegenerative disorders, including Alzheimer's disease (AD) and Parkinson's disease (PD) and multiple sclerosis (Navikas and Link, 1996, McGreer and McGreer, 1999, Venters et al., 1999, Nagatsu et al., 2000, Szczepanik et al., 2000 and McGreer and McGreer, 2001). Microglial cells are involved in the immune surveillance of the central nervous system where they perform an active role in brain inflammation and degenerative diseases (Pyo et al., 1999). Resting microglial cells can become activated during chronic neurodegenerative disorders such as AD, PD and multiple sclerosis (Gonzalez-Scarano and Baltuch, 1999 and Kim and Joh, 2006). Much like astrocytes and microglia, peripheral macrophages become activated by inflammatory mediators in a wide range of degenerative diseases (Navikas and Link, 1996, Nagatsu et al., 2000 and Kucher and Neary, 2005). Both macrophages and microglia are inherently responsive to their extracellular milieu and thus play an important role in the host response. In particular, both cell types are capable of mediating immune responses by the secretion of inflammatory mediators (Klegeris et al., 2005).

The human THP-1 monocytic cell line can be differentiated into macrophages by treatment with phorbol-12-myristate-13-acetate (PMA) (Schwende et al., 1996). In

response to PMA-induced cellular differentiation, macrophages secrete various cytokines such as TNF-α and IL-6, thus depicting an inflammatory response (Sanceau et al., 1995 and Messmer and Reynolds, 2005). In accordance, recent reports have identified sialidase as another important effector protein involved in the immune response (Stamatos et al., 2003, Stamatos et al., 2006 and Nan et al., 2007). It was suggested that the induction of activated T-cells requires the presence of lysosomal sialidase on the cell surface, resulting in the priming of interleukin-4 (IL-4) (Chen et al., 2000). The latest report found endogenous sialidase induced interferon-γ (IFN-γ) production in activated human T lymphocytes (Nan et al., 2007). These results implicate sialidase causing the production of cytokines as a critical step in the immune response. This correlation between increased lysosomal sialidase following cellular stimulation was observed in activated human lymphocytes, CD4 positive T-cells and most recently, THP-1 derived macrophages (Wang et al., 2004, Stamatos et al., 2005 and Nan et al., 2007). Using human THP-1 monocytes, our data suggests that sialidase, like many inflammatory cytokines, can modulate monocyte/macrophage functions, thereby contributing to the release of other inflammatory mediators and possibly, the regulation of the immune response.

Lysosomal sialidase catalyzes the removal of terminal sialic acids from various glycoconjugates, including important surface molecules such as CD44 and CD45 (Gee et al., 2003 and Xu and Weiss, 2002). Recent data has found that upon cell activation via differentiation of human derived monocytes, endogenous sialidase activity is upregulated by 20-30-fold when stimulated with lipopolysaccharide, LPS (Stamatos et al., 2005).

Lysosomal sialidase was shown to be a potent mediator of cellular activation during an immune response and thus provide insight into the physiological processing of this highly multi-functional enzyme. Furthermore, stimulation of human blood monocytes with LPS increased cytokine production, such as TNF-α and IL-6 (Stamatos et al., 2003, Chio et al., 2004 and Bergamaschi et al., 2006). In addition, the expression of proinflammatory cytokines IL-6 and IL-8 were significantly increased in stimulated dendritic cells by lysophopsholipids, showing involvement of the mitogen activated protein kinase (MAPK) signaling pathway (Oz-Arslan et al., 2006). Moreover, IL-6 production was regulated by clyclic adenosine monophosphate (cAMP)-dependent signaling (Bergamashi et al., 2006). The involvement of numerous cytokine proteins is relevant in dissecting the multiple signaling pathways in the inflammation mediated response. In this study, we confirmed that differentiation of human THP-1 derived macrophages using LPS increased TNF-α and IL-6 cytokine production. In addition, we found that overexpression of sialidase amplified TNF-α and IL-6 secretion in differentiated THP-1 macrophages. Inhibition of sialidase activity using the sialidase inhibitor, 2-deoxy-2,3-dehydro-N-acetylneuraminic acid (DANA) resulted in a significant reduction in IL-6 secretion, supporting a role for sialidase during the immune response.

MATERIALS AND METHODS

Cell Culture

A pro-monocytic leukemia cell line, THP-1 monocytes were cultured in Roswell Park Memorial Institute (RPMI-1640) medium supplemented with 10% FBS, 50 units/ml penicillin, 50 μg/ml streptomycin, 500 μg amphotericin B and 4 mM L-glutamine (Invitrogen), incubated at 37°C and 5% atmospheric CO_2. Prior to treatment, cells were counted using the improved Neubauer hemocytomer and plated at 3.0 X 10^6 per 60 mm dish (BD Biosciences). The removal of cell media and 2 washes with 1X PBS, adherent cells were infected with a high titre adenovirus carrying human sialidase (Ad-Sialidase) for 30 minutes in 200 μl PBS (+ 1% MgCl + 1% CaCl) at 37°C. Following infection, adherent cells were supplemented with complete media for 68 hour incubation.

Reagents

THP-1 induced macrophages were stimulated overnight with 2 nM phorbol 12-myrsitate 13-acetate (PMA, Sigma-Aldrich) see Swende et al., 1996. Briefly, THP-1 monocytes were treated with PMA for 16-18 hours at 37°C and 5% atmospheric CO_2. Following overnight treated, non-adherent cells were washed free with 1X PBS and remaining adherent cells were treated with and without varying concentrations of lipopolysaccharide derived from *Escherichia coli* 0111:B4 (Sigma-Aldrich) in low endotoxin RPMI 1640. The sialidase inhibitor 2-deoxy-2,3-dehydro-N-acetylneuraminic acid (DANA) (Toronto Research Chemicals) was used in varying concentrations from

50-1000 μM following 30 minute Ad-Sial infection and allowed to incubate for a further 68 hours at 37°C and 5% atmospheric CO_2.

Cytokine Measurement

Cytokine production in macrophages was determined using enzyme linked immunoassay detection kits after overnight treatment with differing concentrations of bacterial derived LPS (100 ng/ml to 10 μg/ml). Bacterial lipopolysaccharide (LPS) is found on the outer membrane of gram-negative bacterium which is not present on eukaryotic cells; it provides an effective means to elicit an immune response, whereby the activation of pro-inflammatory cytokines ensues (Kitchens et al., 1992, Lien et al., 2000 and Medzhitov, 2001). THP-1 monocytes and THP-1 derived macrophages were washed twice with 1X PBS and then fresh media was replenished, with or without LPS (*Escherichia coli* 0111:B4) purchased from Sigma-Aldrich (St. Louis, MO). During LPS treatment, the culture medium was replaced by media supplemented with 2% low endotoxin FBS. Cell culture medium was collected from triplicate samples following 18-20 hour incubation with the endotoxin at differing concentration (100 ng/ml to 10 μg/ml). Media samples were analysed for human tumour necrosis factor alpha, interleukin-6 and interleukin-4 using the BD OptEIA™ Human ELISA Kit II, (BD Biosciences). The minimum detectable limit of supernatant concentrations of TNF-α was determined to be 2 pg/ml and 4.9 pg/ml for IL-6. The reaction was read in a 96-well microplate reader at emission 450 nm using Safire microplate reader (TECAN).

SDS-PAGE immunoblotting

Following infection with human Ad-Sial, differentiated THP-1 cells were washed twice with 1X PBS on ice and harvested in lysis buffer: 1% Nonidet P-40, 1% deoxycholate, 5mM EDTA, 1mM EGTA, 2mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor cocktail (Roche). The cell lysates was homogenized using 23 ½ G needle and protein concentration was determined by Bradford assay (BioRad). Equal concentrations of protein were subject to sodium-dodecylsulfate-polyacrylamide-gel-electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (PALL). The membranes were then blocked using 5% non-fat milk solution in 1X TBST (100mM NaCl, 150 mM Tris and 0.25% Tween-20, pH 7.4) for 1 hour at room temperature. Antibodies against sialidase and β-actin (ICN Biochemicals) were incubated overnight in 5% non-fat milk blocking solution at 4 °C. Following 5 X 5 minute washes with TBST, nitrocellulose membranes were incubated in horse radish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG, respectively (Amersham Pharmacia). Following another 5 X 5 minute washes, protein was visualized using enhanced chemiluminescence (ECL) from Amersham Pharmacia.

RESULTS

The role of LPS as an inducer of TNF-α and IL-6 release in THP-1 derived macrophages

The differentiation of cultured monocytic cells occurs through the stimulation with phorbol ester, PMA thereby elevating intracellular calcium levels necessary for adherent cells (Tsuchiya et al., 1982 and Schwende et al., 1996). Through differentiation of macrophages, upregulation of cytokines IL-1β and TNF-α expression was observed (Scheibenbogen and Andreesen, 1991). Our results confirm over 25X increase in TNF-α production ($p<0.0008$) found in differentiated THP-1 macrophages versus undifferentiated THP-1 monocytes following overnight treatment with 2 nM PMA (Figure 1). In addition, LPS stimulation of THP-1 monocytes induced significant secretion of TNF-α ($p<1.79E-0.5$) 200-fold over control levels. Following treatment of differentiated THP-1 macrophages with 10 μg/ml LPS, THP-1 cells expressed an 800-fold increase of TNF-α ($p<0.0005$) (Figure 1). In addition, stimulation with LPS had a significant increase in IL-6 secretion in PMA-induced THP-1 macrophages. In particular, macrophages expressed 400X the level of IL-6 following stimulation with 10 μg/ml LPS ($p<1.67E-05$) (Figure 2), but no detectable IL-4 secretion was observed. Overall, the results revealed that treatment with LPS caused increased secretion of both TNF-α and IL-6 in THP-1 derived macrophages.

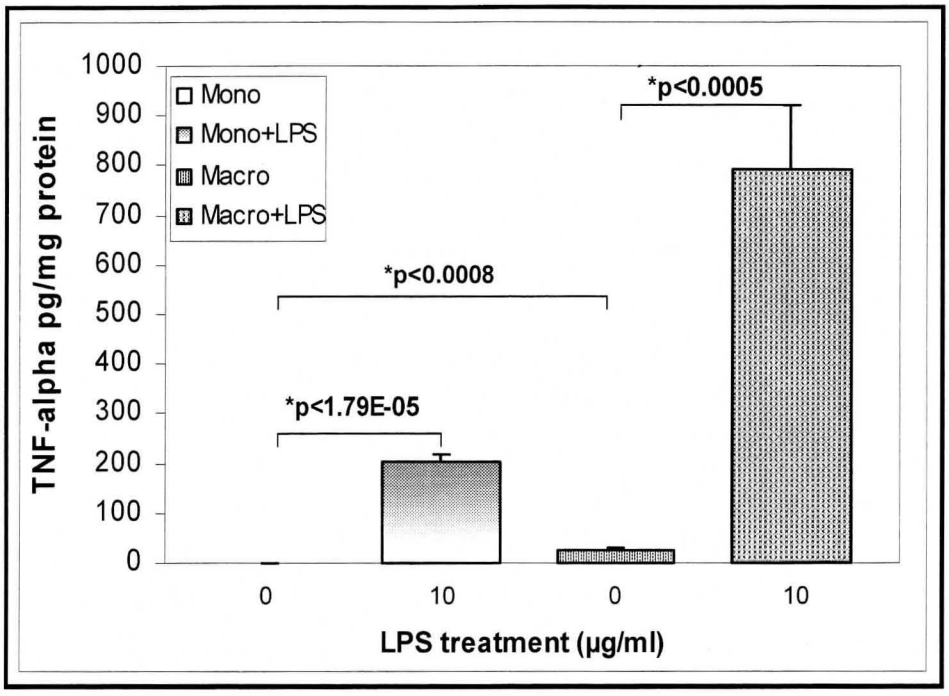

Figure 1. TNF-α secretion in response to lipopolysaccharide in human THP-1 cells. Differentiated THP-1 macrophages were treated overnight with 2nM PMA. Both undifferentiated THP-1 monocytes and differentiated THP-1 macrophages were treated with 10 µg/ml LPS and were incubated at 37 °C for 18 hours prior to human TNF-α ELISA assay. Treatment with LPS induced significant increases of TNF-α production in human THP-1 monocytes and differentiated macrophages. Protein concentration of cell lysates was measured using Bradford protein assay. Data shown represent the mean ± S.D. of triplicate samples and are representative of data from 2 separate experiments.

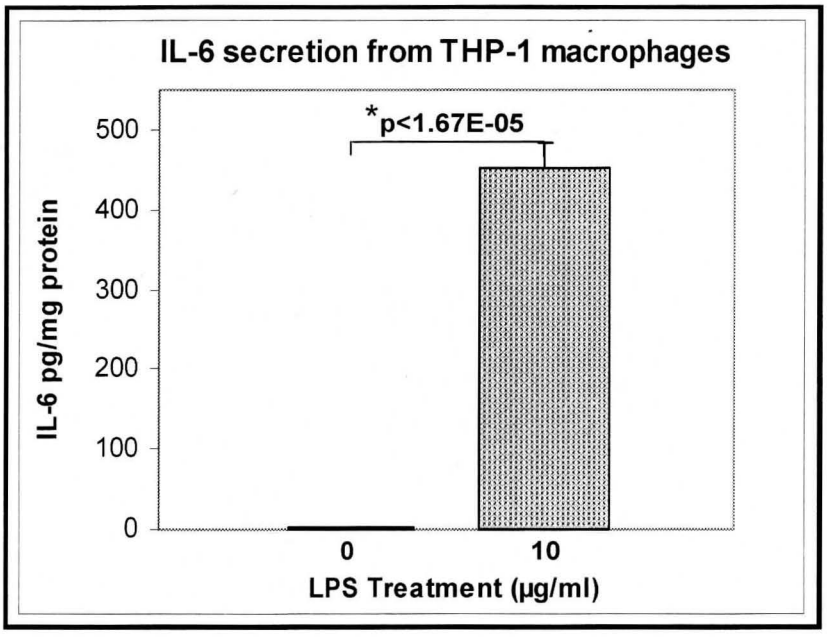

Figure 2. IL-6 secretion in response to lipopolysaccharide in differentiated THP-1 macrophages. THP-1 monocytes were treated with 2nM PMA overnight. Following PMA treatment, differentiated macrophages were stimulated with or without 10 µg/ml of LPS and incubated at 37 °C for 18 hours prior to human IL-6 ELISA assay. Treatment with LPS induced significant increases of IL-6 production in human THP-1 differentiated macrophages. Protein concentration of cell lysates was measured using Bradford protein assay. Data shown represent the mean ± S.D. of triplicate samples and are representative of data from 3 separate experiments.

The role of sialidase as an inducer of TNF-α and IL-6 release in THP-1 derived macrophages:

To investigate the response of these cytokines to sialidase, human THP-1 differentiated macrophages were infected with an adenovirus overexpressing human sialidase and cytokine production was determined using enzyme-linked immunosorbent assay detection kits. Following infection with different levels of Ad-Sialidase, differentiated THP-1 macrophages samples (at 10 MOI) show 200X increase in TNF-α levels ($p<0.0003$) and 500-600X increase at 100 MOI ($p<6.87E-05$) as shown in Figure 3. In addition, human THP-1 macrophages overexpressing sialidase show significantly increased IL-6 levels (Figure 4). Specifically, our data revealed that the expression of pro-inflammatory cytokines, such as TNF-α and IL-6 in differentiated macrophages was induced significantly in response to sialidase overexpression. In addition, our results indicate that sialidase is a potent inducer of TNF-α and IL-6 secretion, but not IL-4 in human THP-1 differentiated macrophages.

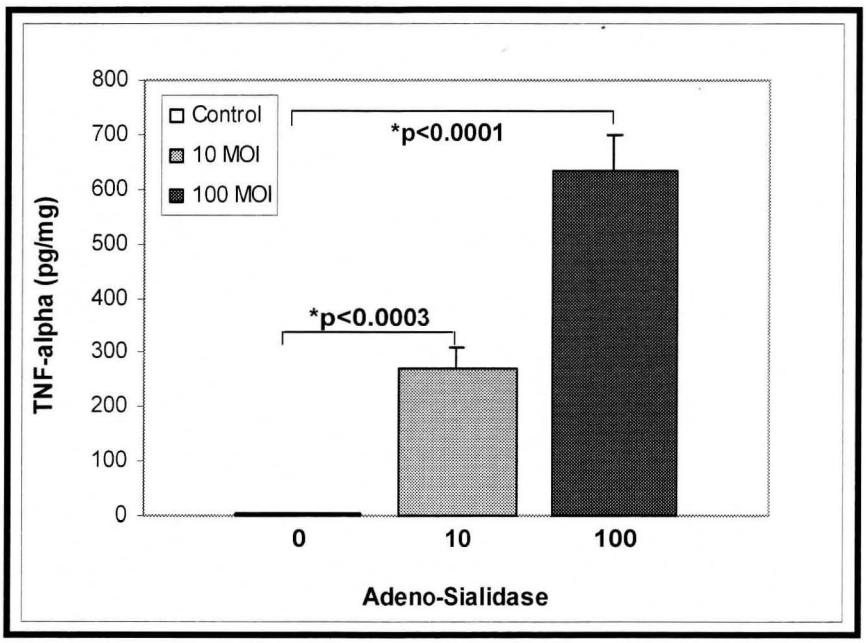

Figure 3. TNF-α secretion in response to sialidase overexpression in human THP-1 cells. Differentiated THP-1 macrophages were treated overnight with 2nM PMA, prior to treatment with 0, 10 and 100 MOI of Ad-Sialidase for 30 minutes, cells were subsequently incubated at 37 °C for 68 hours, and media was collected and measured using human TNF-α ELISA assay. Overexpression of sialidase induced significant increases of TNF-α production in human macrophages. Protein concentration of cell lysates was measured using Bradford protein assay. Data shown represent the mean ± S.D. of triplicate samples and are representative of data from 2 separate experiments.

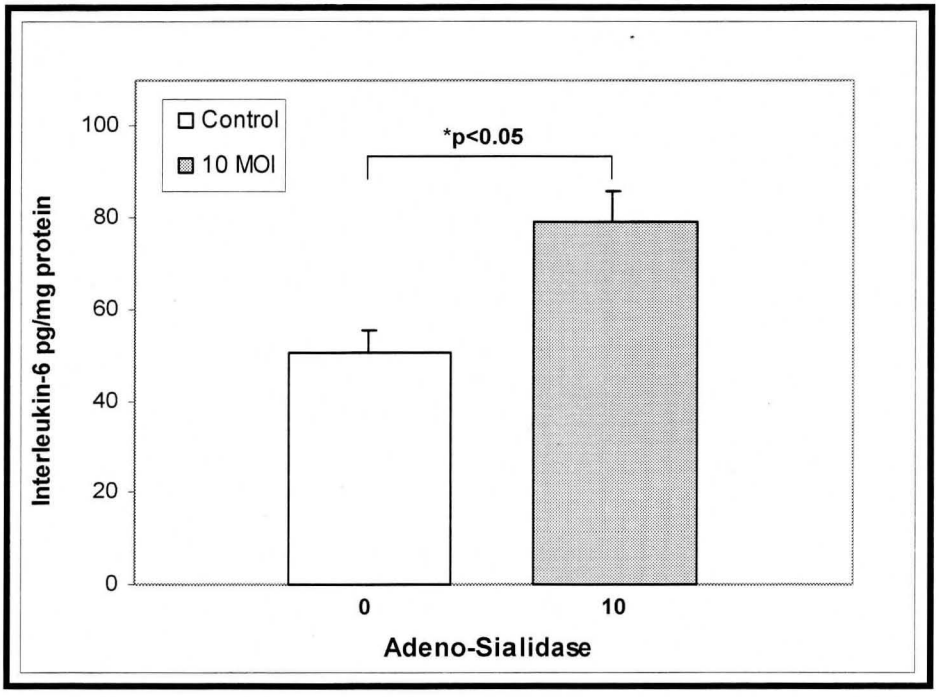

Figure 4. Measuring IL-6 secretion in response to sialidase overexpression in human THP-1 cells. Differentiated THP-1 macrophages were treated overnight with 2nM PMA, prior to treatment with 10 MOI Ad-Sialidase. After 68 hours, media was collected and human IL-6 was measured using ELISA assay. Overexpression of sialidase induced significant increases of IL-6 production in human THP-1 differentiated macrophages. Protein concentration of cell lysates was measured using Bradford protein assay. Data shown represent the mean ± S.D. of triplicate samples and are representative of data from 2 separate experiments.

The sialidase inhibitor, DANA decreases IL-6 secretion and abolishes sialidase expression in differentiated THP-1 macrophages:

To further assess the role of sialidase as an inducer of pro-inflammatory cytokine secretion in human THP-1 macrophages, cells were infected with 50 MOI human Ad-Sialidase and simultaneously treated with variable concentrations (50-1000 μM) of the sialidase inhibitor 2-deoxy-2, 3-dehydro-N-acetyl-neuraminic acid (DANA). The medium of each sample was collected and subject to ELISA for human IL-6 secretion. Our results demonstrate that overexpression of sialidase in differentiated THP-1 macrophages can be achieved using a minimum of 50 MOI of Ad-Sialidase (Figure 5). Figure 6 shows decreased secretion of IL-6 in all samples containing the sialidase inhibitor (50, 100, 500 and 1000 μM) in comparison to the mock-infected sample. Furthermore, western blot analysis of differentiated human THP-1 macrophages overexpressing sialidase in the presence of the sialidase inhibitor (50, 100, 300, 500, 700 and 1000 μM) have shown little sialidase expression (Figure 7). Under these conditions, DANA effectively inhibited the production of IL-6 secretion and abolished sialidase expression.

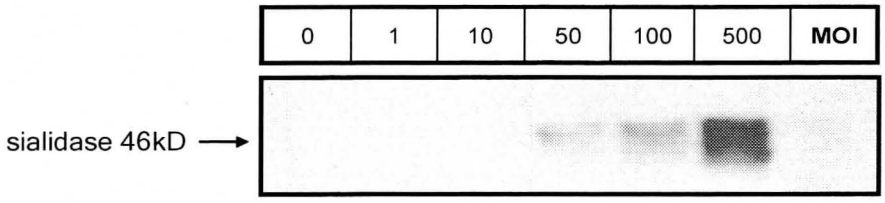

Figure 5. Western blot of macrophages overexpressing sialidase. THP-1 monocytes were treated overnight with 2nM PMA. Adherent macrophages were infected with varying concentrations of Ad-Sial [0, 1, 10, 50, 100 and 500 MOI] for 30 minutes and incubated for a further 68 hours. Samples were harvested and run on 7% resolving gel, transferred to nitrocellulose membrane and incubated overnight with rabbit anti-sialidase antibody. Macrophages show sialidase expression in samples infected with $\geq$ 50 MOI Ad-Sial.

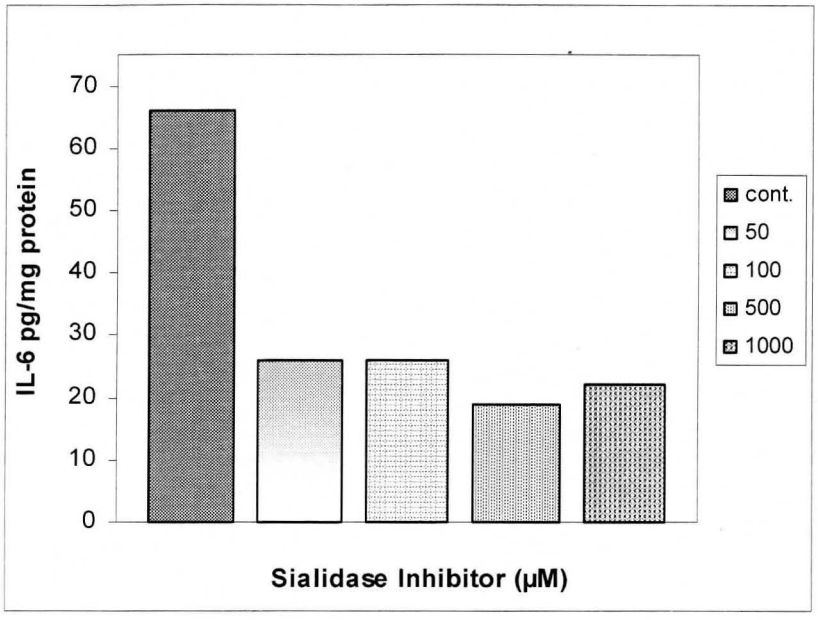

Figure 6. Sialidase inhibitor, DANA reduces sialidase-stimulated IL-6 secretion in THP-1 induced macrophages. Macrophages were infected with 50 MOI Ad-Sialidase for 30 minutes and incubated with different concentrations of the sialidase inhibitor (μM) for 68 hours at 37 °C prior to human IL-6 ELISA assay of the cell culture medium. THP-1 induced macrophages overexpressing sialidase and treated with sialidase inhibitor show reduced IL-6 secretion.

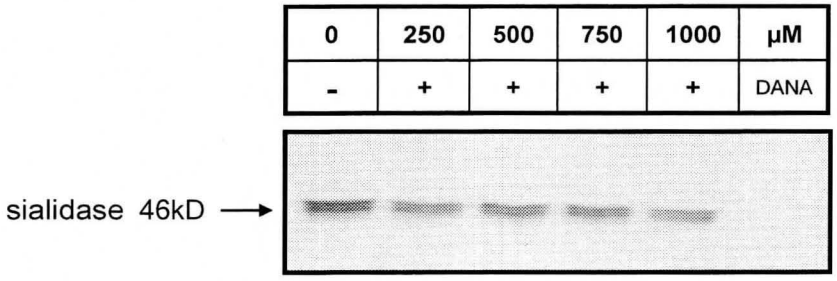

Figure 7. Sialidase inhibitor, DANA reduces sialidase expression in differentiated THP-1 cells. Macrophages were infected with 50 MOI Ad-Sialidase in the presence of varying concentrations of the sialidase inhibitor (µM) and incubated for 68 hours. Samples were harvested and run on 7% resolving gel, transferred to nitrocellulose membrane and incubated overnight with sialidase antibody. Sialidase expression is abolished in THP-1 induced macrophages overexpressing sialidase treated with the sialidase inhibitor DANA.

DISCUSSION

The regulation of circulating cytokines is influenced by a dynamic interplay between proinflammatory agents and neurotransmitters (Raivich et al., 1999). Elevated levels of TNF-α and IL-6 result in the potential to exacerbate the pathogenesis of various inflammatory and neurodegenerative diseases in the injured CNS (Kishimoto et al., 1995 and Van Wagoner and Benveniste, 1999). TNF-α and IL-6 are pleiotropic cytokines involved in many physiological roles in the central nervous system, including immune regulation and inflammation (Bruce et al., 1996, Wada et al., 2000 and Oz-Arslan et al., 2006). We have confirmed that upon stimulation of monocytic cells with LPS, the proinflammatory cytokines TNF-α and IL-6 are upregulated, as previously reported (Stamatos et al., 2003, Chio et al., 2004 and Bergamaschi et al., 2006). In this study, we show that overexpression of sialidase induces both TNF-α and IL-6 release in THP-1 derived macrophages. The increased expression of proinflammatory cytokines IL-6 and TNF-α may elicit the pathogenesis associated with inflammation. Previous work has identified the localization of lysosomal sialidase on the plasma membrane prior to reaching the lysosome, thus shedding light onto the mechanistic sorting of newly generated sialidase (Lukong et al., 2001). Recently, translocation of lysosomal sialidase to the plasma membrane following the differentiation of human blood monocytes was demonstrated (Liang et al., 2006). These targeting studies are important in justifying the role of lysosomal sialidase at the plasma membrane and its potential interaction with surface receptors. In particular, the removal of cell surface sialic acids may unmask and

potentially alter recognition ability and may regulate immune cell interactions. Potential targets are surface glycoproteins CD44 and CD45 which have been shown to be highly modulated by desialylation.

The MAPK signaling pathway is associated with the induction of several proinflammatory mediators, including IL-6 and nitric oxide synthase (iNOS) (Chio et al., 2003 and Oz-Arslan et al., 2006). Recent reports have indicated the involvement of protein kinases phospho-c-jun N-terminal kinase (JNK) signaling pathway associated with IL-6 release (Ciallella et al., 2005 and Oz-Arslan et al., 2006). The production of IL-6 is mediated by numerous transduction pathways and in particular, involves many protein kinases and second messengers including protein kinase A (PKA)-dependent activation of PKC and cAMP (Dendorfer et al., 1994 and Chio et al., 2003). Previous studies have shown that cAMP-dependent signaling was responsible in regulating immune responses in human peritoneal macrophages (Dendorfer et al., 1994 and Williams and Shacter, 1997). The involvement of cAMP is of particular interest because of its involvement in cytokine production by T-cell populations (Chio et al., 2003). Consequently, the activation of these various pathways is associated with the production of IL-6, IL-8, and TNF-α associated inflammation (Ciallella et al., 2005 and Oz-Arslan et al., 2006). In the near future, our group will endeavor to delineate these signaling pathways involved in sialidase mediated IL-6 release in human THP-1 derived macrophages by the use of pharmacologic inhibitors.

The overexpression of endogenous sialidase appears to mediate IL-6 release from human THP-1 macrophages. The carbohydrate compound 2-deoxy-2, 3-dehydro-N-

acetyl-neuraminic acid (DANA) has been shown to bind to the active site on sialidase, thus inhibiting its activity (Bossart-Whitaker et al., 1993). Most surprising was the down regulation of sialidase expression in response to the presence of the inhibitor. Further studies are required to examine the intracellular localization of sialidase after inhibition treatment. Therefore, our results demonstrate for the first time that the inhibition of sialidase can ameliorate the proinflammatory state during an immune response by human THP-1 macrophages. In conclusion, our data clearly indicate that sialidase may play an essential role in amplifying the immune response through stimulation of proinflammatory cytokines TNF-α and IL-6. Under these conditions, the sialidase inhibitor DANA blocked the production of IL-6 secretion and abolished protein expression in differentiated THP-1 macrophages. However, the molecular mechanisms involved in the sialidase-mediated induction of IL-6 expression remain unknown.

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