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Lysosomal sialidase, Neu1: The new role in cell immune response.

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SUMMARY

Sialidases are enzymes that influence cellular activity by removing terminal sialic acid from glycolipids and glycoproteins. Four genetically distinct sialidases have been identified in mammalian cells, each with a predominant cellular localization (lysosomal, cytosolic or plasma membrane-associated) and substrate specificity. In the lysosome, the Neu1 sialidase exists as a component of the multienzyme complex also containing the lysosomal carboxypeptidase A (CathA/protective protein), β-galactosidase and *N*-acetylgalactosamine-6-sulfate sulfatase. The deficiency of Neu1 causes autosomal recessive diseases of children, sialidosis and galactosialidosis. In addition to its role in the intralysosomal catabolism of sialylated glycoconjugates, Neu1 is also involved in cellular signaling during the immune response. In these studies, I showed that during the differentiation of monocytes and the monocytic cell line, THP-1, into macrophages, the majority of Neu1 changes its localization from the lysosomal to the cell surface-associated. In contrast to other cellular sialidases Neu2, Neu3, and Neu4 whose expression either remains unchanged or are down-regulated, the *Neu1* mRNA, protein and activity are specifically increased during the differentiation, consistent with a significant induction of the transcriptional activity of the *Neu1* gene promoter. CathA, which forms a complex with and activates Neu1 in the lysosome, is sorted to the plasma membrane of the differentiating cells similarly to Neu1. Both proteins are first targeted to the lysosome and then are sorted to the LAMP-2-negative, major histocompatibility complex II-positive vesicles, which later merge with the plasma membrane. The inhibition of Neu1 expression with small interfering RNA or with anti-Neu1 antibodies significantly reduced the ability of macrophages to engulf bacteria or to produce cytokines.

To clarify the biological roles of Neu1 and CathA in the immune cells we have further developed animal models of a single CathA deficiency and a double CathA/Neu1 deficiency by gene targeting in mice. Macrophages derived from the splenocytes or the peripheral blood monocytes of the $CathA^{S190A\text{-}Neo}$ mice as well as their immature DCs showed significantly reduced capacity to engulf bacteria. The mature DCs also had lower effect on T cells proliferation, while properties of the cells derived from the $CathA^{S190A}$ mice were indistinct from those of the wild type controls. Macrophages from the Neu1-deficient mice showed an increased affinity to FITC-labeled *Maackia amurensis* lectin, consistent with a compromised processing of sialylated sugar residues of cell surface molecules. Altogether our data suggest that the cell surface Neu1 plays an important role in the regulation of affinity of the immune cells towards each other and external pathogens.

We also show that the upregulation of the Neu1 expression is important for the primary function of immune cells and establish the link between Neu1 and the cellular immune response.

Key words: Neu1, sialidase, CathA, monocyte differentiation, cell signaling, immune response, lysosomes

RÉSUMÉ

Les sialidases sont des enzymes qui agissent sur l'activité cellulaire en enlevant l'acide sialique terminal des glycolipides et des glycoprotéines. Quatre sialidases génétiquement distinctes on été identifiées dans les cellules de mammifères, chacune ayant une localisation cellulaire (lysosomale, cytosolique ou associée à la membrane plasmique) et une spécificité de substrat prédominantes. Dans le lysosome, la sialidase Neu1 fait partie d'un complexe multienzymatique auquel appartiennent aussi la carboxypeptidase A lysosomale (CathA/protéine protectrice), la β-galactosidase et la *N*-acetylgalactosamine-6-sulfate sulfatase. La déficience en Neu1 cause des maladies autosomales récessives chez l'enfant : la sialidose et la galactosialidose. En plus de son rôle dans le catabolisme intralysosomal des composés glycoconjugués sialilés, Neu1 est aussi impliqué dans la signalisation cellulaire pendant la réponse immunitaire. Dans ce projet, j'ai démontré que lors de la différenciation en macrophages des monocytes et de la lignée monocyte THP-1, la majeure partie de Neu1 devient associée à la surface de la cellule plutôt que lysosomale. Contrairement à d'autres sialidases cellulaires, Neu2, Neu3 et Neu4, dont l'expression reste soit inchangée ou est régulée à la baisse, l'ARNm, la protéine et l'activité de Neu1 sont spécifiquement augmentés pendant la différenciation, allant de pair avec une induction significative de l'activité transcriptionnelle à partir du promoteur Neu1. CathA, qui fait partie du même complexe et qui active Neu1 dans le lysosome, est aussi envoyé vers la membrane plasmique des cellules en différenciation, tout comme Neu1. Les deux protéines sont d'abord envoyées vers le lysosome puis sont triées dans des vésicules LAMP-2 négatif / complexe majeur d'histocompatibilité II positif, qui se fusionnent ensuite à la membrane plasmique. L'inhibition de l'expression de Neu1 avec de petits ARN d'interférence ou avec des anticorps anti-Neu1 réduit de façon significative la capacité des macrophages à engloutir des bactéries ou à produire des cytokines.

Afin de clarifier les rôles de Neu1 et CathA dans les cellules immunitaires, nous avons développé par ciblage de gènes des modèles de souris possédant une

déficience simple pour CathA et une double déficience CathA/Neu1. Les macrophages dérivés des splénocytes ou des monocytes du sang périphérique des souris *CathA*$^{S190A\text{-}Neo}$, tout comme leurs cellules dendritiques immatures, montrent une réduction significative de leur capacité à engloutir les bactéries. Les cellules dendritiques matures ont aussi montré un effet diminué sur la prolifération des lymphocytes T, alors que les propriétés des cellules dérivées des souris *CathA*^{S190A} sont indistinctes de celles des contrôles de type sauvage. Les macrophages provenant de souris déficientes en Neu1 ont montré une affinité accrue envers la lectine de *Maackia amurensis* couplée au FITC, ce qui est conséquent avec une entrave au traitement des résidus de sucre sialylés des molécules de la surface cellulaire. En somme, nos données suggèrent que le Neu1 situé à la surface de la cellule joue un rôle important dans la régulation de l'affinité des cellules immunitaires entre elles et envers les pathogènes externes.

Nous avons aussi démontré que la régulation à la hausse de l'expression de Neu1 est importante pour la fonction primaire des cellules immunitaires et établi le lien entre Neu1 et la réponse immunitaire cellulaire.

Mots clés : Neu1, sialidase, cathepsine A, différenciation des monocytes, signalisation cellulaire, réponse immunitaire, lysosomes.

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LIST OF ABBREVIATIONS

APC	Antigen presenting cell
4MU-NeuAc	4 methylumbellifeeryl-alpha-D-N-acetylneuramic acid
CathA	Cathepsin A
DAPI	4, 6-diamidino-2-phenylindole
DMSO	Dimethylsulfoxid
DNA	Dinucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
GALNS	N-acetylgalactosamine-6-sulfatase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GS	Galactosialidosis
HEX	Hexosaminidase
KDa	Kilo Dalton
LAMP	Lysosomal associated membrane protein
LAP	Lysosomal acid phosphate
LIMP	Lysosomal integral membrane protein
LSD	Lysosomal storage disease
Neu1	Lysosomal sialidase
Neu2	Cytosolic sialidase
Neu3	Plasma-membrane-associated sialidase
PAGE	Polyacrylamide gel electrophoresis
PMA	12-O-tetradecanoylphorbol-13-acetate
PBS	Phosphate-buffered-saline
SDS	Sodium dodecyl sulfate
SL	Sialidosis
TBS	Tris buffered saline

AMINO ACID CODES

Name	Three-letter Code	One-letter Code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

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CHAPTER 1: GENERAL INTRODUCTION

1.1 Lysosomes: biogenesis and biological role

Lysosomes are cytoplasmic organelles discovered in 1949 by a Belgian biologist Christian de Duve. They harbor over 100 hydrolytic enzymes such as nucleases, proteases, lipases, glycosidases, phosphatases, sulfatases and phiospholipases. All of these enzymes have an acidic pH optimum and are essentially involved in the degradation of all types of biological macromolecules including lipids, polysaccharides, proteins, nucleic acids, and even whole bacteria and worn out and nonfunctioning organelles like the mitochondria.

The main pathways in the biogenesis of lysosomes are shown in **Figure 1**. The first pathway is followed by macromolecules from receptor-mediated endocytosis. Initially, the endocytosed material, including the receptors, ligands and associated membrane, is delivered into small irregularly shaped intracellular vesicles, so-called early endosomes. Some of the ingested molecules are then selectively retrieved and recycled to the plasma membrane, while the rest of the vesicles become late endosomes. Next, late endosomes fuse with the vesicles containing lysosomal hydrolases from the Golgi apparatus to form mature lysosomes. At a lower pH, lysosomes favor the release and the hydrolytic digestion of the endocytosed molecules. A second pathway can degrade worn-out organelles such as mitochondria in a process called autophagy. In this process, an organelle is enclosed by membranes derived from the ER, producing an autophagosome which then fuses with vesicles containing lysosomal enzymes and become mature lysosome. The third pathway (called phagocytosis) involves the uptake and degradation of large particles and microorganisms like bacteria by specialized cells (phagocytes). These phagocytes (macrophages and neutrophiles in vertebrates) engulf objects to form phagosomes, a counterpart to autophagosomes. The phagosomes are later converted to lysosomes.

As described above, the biogenesis of lysosomes is a complex process that requires that specific sets of soluble hydrolases and membrane proteins, synthesized in the rough endoplasmic reticulum (RER), be segregated from proteins with other sub-cellular destinations and be transferred to form mature lysosomes. Any failure in the biogenesis of lysosomes due to genetic mutation can lead to a severe inherited

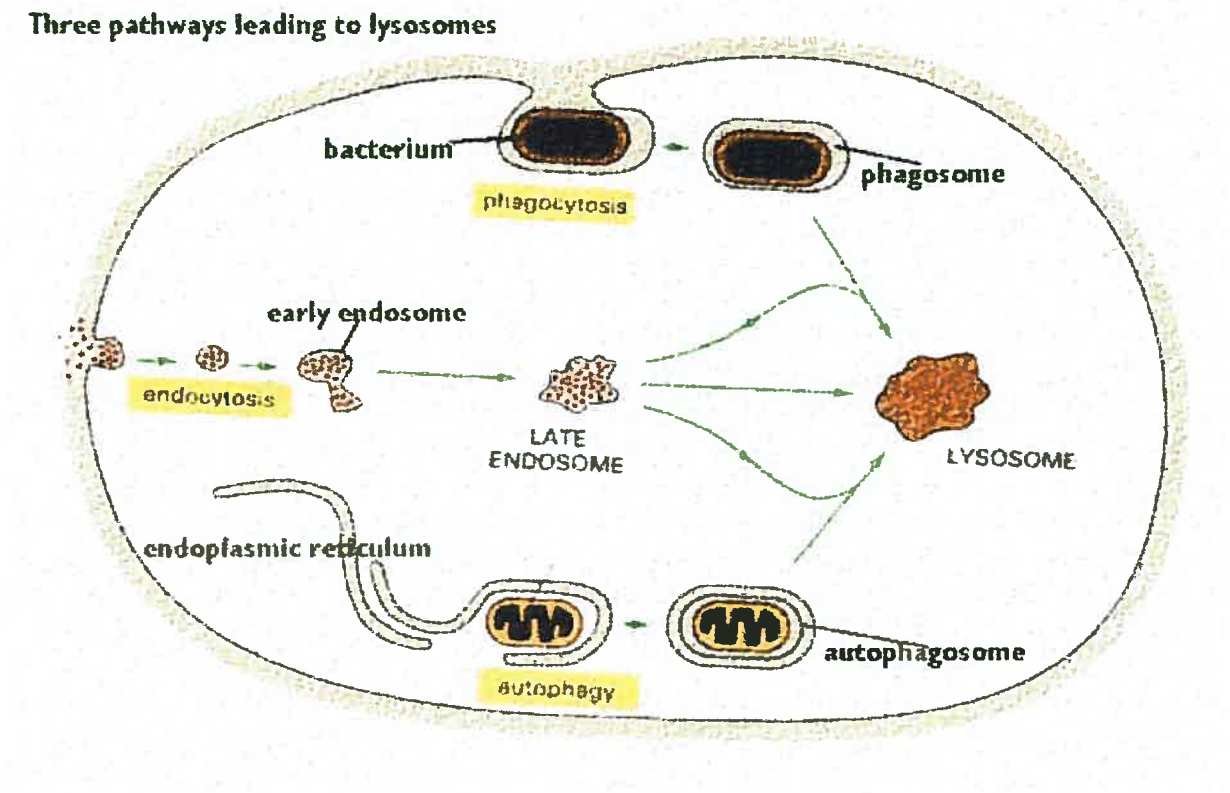

Figure 1. Biogenesis of lysosomes. (Molecular Biology of the Cell by Bruce Alberts, Dennis Bray, Julian Lewis, Martin Raff, Keith Roberts and James D. Watson, Garland Publishing, NY 1994).

Figure 1

disorder, featured by the absence of one or more lysosomal enzymes. These disorders are called lysosomal storage disorders because they result from the accumulation of undigested macromolecules in the lysosomes of the affected tissues.

In 1967, Leroy and DeMars identified large, phase-dense inclusions in the fibroblasts of a patient with a disorder harboring a clinical resemblance to a Hurler Syndrome (Leroy et al., 1967). Since these cells were called inclusion cells (I-cells), the disorder later became known as I-cell disease (mucolipidosis II). In 1972 Hickman and Neufeld found that multiple deficiency of lysosomal enzymes in I-cell disease results from a deficiency in a recognition marker, which is common to all soluble lysosomal enzymes and is required for their targeting to the lysosomes. This discovery eventually led to the identification of the lysosomal targeting marker of the soluble lysosomal proteins, mannose-6-phosphate (Kaplan et al., 1977) and its two distinct receptors of 275-300 kDa and 46 kDa, respectively. Intrinsically different mechanisms have later been identified for the targeting of membrane-bound lysosomal enzymes (reviewed in Neufeld, 1991; Natowicz et al, 1979).

1.1.1 Lysosomal membrane

The lysosomal membrane, equipped with carriers, transport systems and a proton pump that maintains the pH of the lumen, is serving as a selective permeability barrier between the lysosomal lumen and the cytoplasm. It prevents macromolecules that enter the lysosomes by endocytosis or autophagy from egress, but allows exit of certain small molecules and degradation products. Monosaccharides seem to cross the membrane by carrier-mediated systems. For example, sialic acid transfer is mediated by a specific carrier, sialic acid transporter protein (Havelaar et al., 1999). A deficiency in this carrier causes Salla disease, characterized by sialic acid accumulation within the lysosomes (Martin et al. 2003). Sanfilippo C syndrome is another disease caused by a defect in a lysosomal membrane transporter, acetyl-CoA: alpha glycosaminide N-acetyltransferase (Hrebicek et al., 2006), which is normally involved in the transmembrane acetylation of heparan sulfate. Since acetylation is necessary for further heparan sulfate

degradation in Sanfilippo C syndrome, heparan sulfate accumulates in the lysosomes (Bame and Rome, 1985).

Using polyclonal and monoclonal antibodies prepared against the lysosomal membrane, several other integral or associated membrane proteins have been identified, including lysosomal-associated membrane proteins (LAMPs) and lysosomal integral membrane proteins (LIMPs) (reviewed in Hunziker and Geuze, 1996). These proteins are mostly found on the lumenal side of the lysosomal membrane and contain many complex oligosaccharides bearing sialic acid residues, which are thought to protect the membrane from the attack of lysosomal hydrolases. The sialic acid moieties may also contribute to the establishment of a Donnan potential for protons that maintains to the low pH of the lysosomes (Cretin, 1982).

1.1.2 Biosynthesis of lysosomal enzymes

1.1.2.1 Sorting of lysosomal membrane proteins

Membrane-bound lysosomal proteins are normally glycoproteins rich in N-linked oligosaccharides, most of which are complex. These proteins are synthesized and glycosylated in the RER and their oligosaccharides modified in the Golgi apparatus.

The sorting of membrane-bound lysosomal proteins, such as LIMPs and LAMPs (Barriocanal et al., 1986), involves their transportation to the lysosomes by a mechanism requiring the association of Gly-Tyr-X-X-hydrophobic Leu-leu amino acid motifs at the carboxyl terminus of their cytoplasmic domain with μ-subunit of HA adaptor complexes (Guarnieri et al., 1993; Pearse et al., 2000). It is independent of mannose-6-phosphate receptors which mediate transport of soluble lysosomal enzymes. The same pathway is used by the lysosomal acid phosphatase (LAP), which is synthesized and transported to lysosomes as a transmembrane protein (Braun et al., 1989). In lysosomes, LAP is released from the membrane by proteolytic processing, which involves at least two cleavages at the C-terminus of LAP. One cleavage is catalyzed by a thiol proteinase outside the lysosomal membrane, removing the bulk of the cytoplasmic tail of LAP. Another cleavage is catalyzed by an aspartyl

proteinase inside the lysosomes and releases the luminal part of LAP from the membrane-spanning domain (Gottschalk et al., 1989).

Existence of a distinct sorting mechanism for the membrane-associated lysosomal proteins is further supported by the presence of normal levels of β-glucocerebrosidase and acid phosphatase in I-cell fibroblasts. Moreover, pulse-labeling and cell fractionation experiments of rat kidney cultured cells treated with the glycosylation inhibitor, tunicamycin, have indicated that newly synthesized plasma membrane proteins lacking N-linked oligosaccharides were nonetheless rapidly transported to the lysosome (Barriocanal et al., 1986). These studies with tunicamycin confirmed that the sorting of lysosomal membrane proteins is different from the transport of soluble lysosomal enzymes.

1.1.2.2 Sorting of soluble lysosomal proteins

Significant progress has been made towards an understanding of the targeting mechanisms of newly synthesized soluble lysosomal enzymes. Lysosomal proteins as well as most secretory proteins are glycoproteins synthesized in polyribosomes bound to the RER membranes. Each protein contains an amphyphilic signal peptide at the N-terminus which interacts with a signal recognition particle, an 11 S ribonucleoprotein, thereby initiating the vectoral transfer of the nascent protein across the RER membrane into the lumen of this organelle (Erickson et al., 1981). In the initial stage of glycoprotein synthesis, a large precursor oligosaccharide $Glc_3Man_9GlcNAc_2$ (three glucose, nine mannose and two N-acetylglucosamine residues), is assembled with a pyrophosphate link to a ligand carrier, dolichol (Dol), and then transferred to the target asparagine residue of the precursor glycoprotein. The signal peptide is cleaved still in the RER, followed by processing of the asparagine-linked (or N-linked) oligosaccharide. Glucosidases I and II excise three glucoses, and mannosidase cleaves one of the mannose residues from the sugar chain.

Then the proteins move to the Golgi stack by vesicular transport where they undergo a number of post-translational modifications and are sorted for targeting to the further destination: the lysosome, secretory granule or plasma membrane. The

Golgi complex consists of a stack of flat cisterns functionally divided into three regions: *cis*, medial and trans Golgi. The *cis* part normally receives products of biosynthesis from the RER. During the passage through the Golgi apparatus, the oligosaccharide chain residues on secretory and membrane glycoproteins are processed stepwise to complex-type units containing sialic acid. This processing removes five mannose residues and adds 3 molecules: N-acetylglucosamine, galactose and sialic acid.

Almost all of the oligosaccharides on lysosomal enzymes undergo a different series of modifications that introduce the Man-6-P marker. The Man-6-P distinguishes lysosomal from secretory glycoproteins and is responsible for addressing them via one of two Man-6-P receptors to their lysosomal destinations. The Man-6-P marker is incorporated into lysosomal enzymes by a two-step process that requires the sequential actions of the two enzymes. The first step involves the enzyme UDP-GlcNAc:lysosomal enzyme N-acetylglucosamine 1-phosphotransferase (phosphotransferase), a multi-subunit protein (Bao et al., 1996; Raas-Rothschild et al., 2000) which transfers N-acetylglucosamine 1-phosphate (GlcNAc-1-P) from the nucleotide sugar uridine diphosphate-N-acetylglucosamine (UDP-GlcNAc) to the C-6 hydroxyl position of specific mannose residues on the high mannose oligosaccharides of newly synthesized lysosomal enzymes, to produce a phosphodiester intermediate. This modification of mannose residues in the *cis*-Golgi protects them from cleavage by mannosidases in the medial Golgi. In the medial Golgi, the second enzyme, a specific phosphodiesterase called N-acetylglucosamine-1-phosphodiester α-N-acetylglucosaminidase (phosphodiesterase) (Kornfeld et al., 1999) removes the terminal N-acetylglucosamine (GlcNAc) residue uncovering the phosphate, thus exposing the phosphomannosyl signal. Subsequently, the lysosomal hydrolases bind to the mannose-6-phosphate receptors in the trans-Golgi network, thus remaining intracellularly.

Two known Man-6-P receptors differ in their binding properties and divalent cation requirements (Hoflack et al., 1985). The first identified one is a 275-300 kDa cation-independent Man-6-P receptor (CI-MPR, MPR300), which is a type I

transmembrane glycoprotein with a segment of 17 kDa exposed on the cytoplasmic side of the membrane. Detected in the Golgi complex, coated vesicles, endosomes, and the plasma membrane, CI-MPR participates in the endocytosis of extra-cellular Man-6-P-containing proteins and has an insulin-like growth factor type II receptor function. The importance of CI-MPR for lysosomal enzyme trafficking was demonstrated by the massive mistargeting of lysosomal enzyme precursors in homozygote embryos of chimeric mice lacking CI-MPR (Sahagian et al., 1984).

The second receptor is a glycoprotein composed of three subunits of 46 kDa each (Hoflack et al., 1985 and Robbins et al., 1981). As the cation-dependent Man-6-P receptor (CD-MPR, MPR46), CD-MPR was first discovered in the endothelial cells. While the CI-MPR appears to have a dominant role in lysosomal targeting, the function of CD-MPR remains unclear. Like CI-MPR, CD-MPR is a transmembrane protein with a cytoplasmic domain of 69 residues. Both CI-MPR and CD-MPR, like other integral membrane proteins and endocytosed receptors, have sorting determinants in their cytoplasmic domains. The trafficking of CI-MPR is mediated by the tyrosine- and di-leucine-based motifs, while the CD-MPR has two distinct targeting peptides: a weak YRGV sequence and a dominant FPHLAF sequence (Collawn et al., 1991; reviewed in Le Borgne and Hoflack, 1998; Schweizer et al., 2000). The affinity of these receptors to Man-6-P moieties of lysosomal hydrolases is pH dependent: the strongly acidic lysosomal medium favors dissociation of the ligand (Dahms et al., 1989), whereas at neutral or slightly acidic pH, the receptors bind strongly to their ligand. Both receptors are recycled back to the Golgi complex through the interaction with so-called tail-interacting 47-kDa protein (TIP47), which binds the cytoplasmic domains of the CD-MPR and CI-MPR and mediates their transport from endosomes to the Golgi complex (Orsel et al. 2000).

1.2 Mammalian neuraminidases (sialidases)

Sialidases are widely distributed in vertebrates as well as in microorganisms such as viruses, bacteria, fungi and protozoa, most of which are unable to produce sialic acid themselves, but must process sialic acids on their hosts in order to infect

and replicate (Miyagi et al., 1993; Warner et al., 1993, Colman 1994; Schenkman et al., 1994; Chou et al., 1996). Sialidases are glycosidases that hydrolyze the alpha-2,3-, alpha-2,6- and alpha-2,8 linkages of terminal sialic acid residues in various sialoglycoconjugates including oligosaccharides, glycoproteins, glycolipids, colominic acid and synthetic substrates. They are also known as exo-alpha sialidases, N-acylneuraminate glycohydrolases, N-acetyl neuramyl hydrolases or neuraminidases (EC 3.2.1.18)

In vertebrates, sialidases modulate cellular events such as activation, differentiation, maturation and growth, all of which involve the changes of sialic acid level (Schauer T, 1985). In microorganisms, sialidases are believed to be important in nutrition, using liberated sialic acid as a source of energy (Corfield, 1992). In other situations, the bacterial sialidases are involved in pathogenesis. For example, *Vibrio cholerae* sialidase removes sialic acid from the gangliosides on the surface of interstinal cells to produce G_{M1}-ganglioside, the binding site for cholera toxin (Galen, 1992; Corfield, 1992; Tang et al., 1996).

In mammals, 4 distinct sialidases encoded by *Neu1-Neu4* genes have been identified. They differ in their subcellular localization, substrate preference and pH optimum (Carrillo et al., 1997, Monti et al., 1999, Monti et al., 2000 and Seyrantepe et al., 2004).

1.2.1 Cytosolic sialidase (Neu2)

Cytosolic sialidase was first isolated from rat skeletal muscles and Chinese hamster ovary cells and found to be optimally active at pH 6.0 (Miyagi et al., 1990, 1993; Sato and Miyagi, 1995). Monti et al. (1999) identified the human cytosolic sialidase gene, named Neu2 and located at human chromosome 2 (2q37), using a sequence homology-based approach. The gene encodes a 380-aa polypeptide with a deduced molecular mass of 42 kDa, containing two Asp blocks, a conserved amino acid consensus sequences (G-X-D-X-G-X-X-W/F) that are called Asp boxes and found in all sialidases. Another conserved sequence YRIP that contains an active site nucleophyl is found in the amino terminal part of the peptide. Neu2 has a broad

substrate specificity and is active against αÁ → -sialylated oligosaccharides, glycopeptides, and some gangliosides including G_{M3}, G_{D1a} and G_{D1b}-gangliosides, but not G_{M1} and G_{M2}-gangliosides as well as the artificial fluorogenic substrate, 4 methylumbelliferyl-alpha-D-N-acetylneuramic acid (4MU-NeuAc) (Miyagi and Tsuiki, 1985). The exact biological role of Neu2, which is expressed mostly in muscle cells especially during their diffentiation, is still unknown (Sato and Miyagi, 1996; Akita et al., 1997), however, it was suggested that this enzyme may lead to the alteration of cytoskeletal functions, through cleaving G_{M3}-ganglioside that is associated with the cytoskeleton. The Neu2 activity of melanoma cells inversely correlates with their invasive and metastatic potential (Tokuyama et al., 1997).

1.2.2 Plasma membrane sialidase (Neu3)

Plasma membrane sialidase, also referred to as G_{M1}-ganglioside sialidase, G_{M1} sialidase and ganglioside sialidase, is an integral membrane protein with a strict specificity for gangliosides such as G_{M1}, G_{D1a} and other polysialogangliosides (Schneider-Jakob and Cantz, 1991) but not glycoproteins or oligosaccharides (Kopitz et al., 1997; Miyagi et al., 1999). Miyagi et al. (1999) cloned and expressed a ganglioside sialidase cDNA, isolated from a bovine brain cDNA library. The enzyme encodes a 428-aa protein with a deduced molecular mass of 47.9 kDa and contains a transmembrane domain and three Asp boxes. Neu3 has a pH optimum of 4.6 and is activated by non-ionic detergents such as Triton X-100. Neu3 has been recognized as an important modulator of cellular functions including cell-cell and cell-matrix interactions, cell proliferation, differentiation and oncogenic transformation (Hakomori and Igarashi., 1993; Kopitz et al., 1996, 1998). In ganglions, the enzyme is probably involved in neuritogenesis, synaptogenesis and neuronal survival (Tettamanti and Robin, 1993; Kopitz et al., 1994, 1997). Monti et al. (2000) cloned cDNA of human plasma-membrane-associated sialidase (Neu3), which has a 78% sequence homology to the bovine protein (Miyagi et al., 1999). Human Neu3 is a 428-aa protein highly active against ganglioside substrates, and has a pH optimum of 3.8.

1.2.3 Human lysosomal sialidase (Neu1)

1.2.3.1 Biochemical properties

Lysosomal sialidase, Neu1 has pH optimum of 5.0 and catalyzes the removal of terminal sialic acid residues from oligosaccharides, some glycolipids and short glycopeptides, but is inactive against glycoproteins such as fetuin or submaxillary mucin (Miyagi and Tsuiki, 1985, Schneider-Jakob and Cantz, 1991, Hiraiwa et al. 1987, 1988). In the lysosomes the release of the sialic acid triggers further degradation of the sugar moiety by other glycosidases. The human enzyme preferentially cleaves αÁ-3 and αÁ-6 sialyl bonds in sialoconjugates (Frisch and Neufeld, 1979). Early reports suggested that in addition to soluble (luminal) sialidase, lysosomes also contain another enzyme, associated with their membranes. Although this enzyme has never been isolated, Miyagi et al. (1990) reported that the lysosomal membrane sialidase from rat liver has an acidic pH optimum like its plasma membrane counterpart and a broad specificity in hydrolyzing gangliosides as well as fetuin, sialyllactose and 4MU-NeuAc (Kopitz et al., 1996). Since Neu1 is often completely deficient in the cells of patients affected by the inherited disease, sialidosis caused by the mutations in the Neu1 gene (Bonten et al., 1996; Pshezhetsky et al., 1997), both lysosomal membrane and intralysosomal sialidases are probably encoded by the *Neu1* gene.

Multiple attempts, to purify and characterizate Neu1 did not succeed because of a low tissue content and an extreme liability of the enzyme. Verheijen et al. (1983, 1985) indicated that the lysosomal carboxydase A (CathA, protective protein) forms a complex with and activates Neu1 in bovine testis. Further experiments showed that in the lysosomes of mammalian tissues three glycoproteins: CathA, β-galactosidase and Neu1 form a multi-enzyme complex. Morreau et al. (1992) suggested that β-galactosidase and CathA associate soon after synthesis in the ER and co-migrate to the lysosomes, where they acquire their active and stable conformations. All 3 components of the complex can be co-purified on both β-galactosidase-binding or CathA-binding affinity matrices (Potier et al., 1990; Pshezhetsky and Potier, 1994).

In particular, Pshezhetsky and Potier (1994, 1996) purified human placental CathA on agarose-Phe-Leu affinity column and concluded that CathA forms 1,270-kDa complex with CathA, β-galactosidase and the lysosomal N-acetylgalactosamine-6-sulfatase (GALNS). Only a small percentage of total CathA and β-galactosidase occur in the complex, which however contains all Neu1. This in turn explains why it is impossible to isolate Neu1 activity separately from the complex (Hoogeveen et al., 1983; Warner et al., 1993; Hubbes et al., 1992).

1.2.3.2 Activity and Specificity

The involvement of Neu1 in the hydrolysis of sialilated gangliosides has been a subject of debate for a long time. Characterization of the storage products in urine and cultured fibroblasts from sialidosis patients revealed that sialylated oligosaccharides are a major natural substrate for Neu1 (Strecker et al., 1977; Dorland et al., 1978, van Pelt et. al., 1988), whereas the results on the analysis of the storage products in the autopsy materials from sialidosis patients contradicted these findings. For some patients, several fold increase of G_{M3} and G_{D3} gangliosides was observed in systemic organs (Ulrich-Bott et al., 1987) and brain (Yoshino et al., 1990), but other studies performed on the cells from sialidosis patients (Sakuraba et al., 1983) or in the knock-out mouse model (Zhou et al., 1995) did not confirm storage of gangliosides. The fact that the cultured fibroblasts of sialidosis and galactosialidosis patients treated with radioactively labeled G_{M1}-ganglioside accumulated G_{M3}-ganglioside (Mancini et al., 1986) also suggested that Neu1 is involved in degradation of this glycolipid.

Further studies (Schneider-Jakob and Cantz, 1991, Hiraiwa et al. 1987, 1988) showed that the hydrolysis of gangliosides by Neu1 depends on presence of ionic detergent, such as sodium cholate or taurodeoxycholate (Triton X-100 that activated Neu3 does not have effect on Neu1), suggesting that in vivo this reaction requires activator proteins that play in the lysosome a role of natural detergents (Conzelmann, 1987). This idea was confirmed by Fingerhut et al. (1992), who showed that Neu1 cleaved G_{M3}, G_{D1a}, and G_{T1b}-gangliosides in the presence of Saposin B (also called

sulfatide activator protein). The complete hydrolysis of G_{D1b}-ganglioside to lactosylceramide by glycoprotein fraction from human placenta containing essentially all soluble lysosomal enzymes required the presence of two activators, Saposin B that activated reactions catalyzed by β-galactosidase and Neu1 and G_{M2}-activator that activated reaction of G_{M2} to G_{M3} conversion by hexosaminidase A. Gangliosides G_{D1b}, G_{M1} and G_{M2} were extremely poor substrates for the Neu1 (Fingerhut et al., 1992). The last conclusion was however later reconsidered when asialylated G_{M1} and G_{M2} gangliosides, G_{A1} and G_{A2}, respectively were found among major storage products in knock-out mouse models of G_{M1}-gangliosidosis (Matsuda et al., 1997) and Sandhoff disease (combined deficiency of hexosaminidases (HEX) A and B) (Huang et al., 1997).

Sandhoff mice succumbed to a profound neurodegenerative disease by 4-6 months of age, resembling human phenotype, while Tay-Sachs mice depleted of hexosaminidase A remained asymptomatic to at least 1 year of age because G_{A2}-ganglioside was further efficiently cleaved by hexosaminidase B (Huang et al., 1997). Therefore, mouse models of Tay-Sachs disease have revealed a metabolic bypass of the genetic defect based on the more potent activity of Neu1 towards G_{M2}. In order to determine whether a similar effect would be produced by increasing the level of Neu1 in human Tay-Sachs cells, Igdoura et al (1999) introduced a human *Neu1* cDNA into neuroglia cells derived from a Tay-Sachs fetus and demonstrated a dramatic reduction in the accumulated G_{M2}. These studies proved involvement of Neu1 in the hydrolysis of G_{M2}, suggesting a new method for the treatment of human Tay-Sachs disease.

1.2.3.3 Cloning and characterization of human Neu1

Using the bacterial conserved sequences and taking advantage of the rapid progress of the Human Genome Project, several groups simultaneously cloned and sequenced human (Bonten et al., 1996; Pshezhetsky et. al., 1997; Milner et al., 1997) and mouse (Carillo et al., 1997; Igdoura et al., 1998, Rottier et al., 1998) Neu1. Bonten et al. (1996) identified an expressed sequence tag (EST) clone containing a

full-length *Neu1* cDNA. The mRNA was highly expressed in the pancreas, kidney and skeletal muscle, but showed low expression level in the brain. Similarly, Pshezhetsky et al. (1997) searched the expressed sequences tags database (dbEST) for human analogues of bacterial sialidases and found several overlapping clones from human fetal brain, spleen and placenta. Using primer complementary to the sequences of two overlapping clones, they obtained a complete cDNA by RT-PCR amplification. Both Bonten et al. (1996) and Pshezhetsky et al. (1997) localized the gene by in situ hybridization on chromosome 6p21.3 within the human major histocompatibility complex as previously suggested by Oohira et al. (1985).

The *Neu1* cDNA contains an open reading frame (ORF) of 1245 nucleotides, encoding a protein of 415 amino acids. The first 47 amino acids represent the signal peptide consisting of a positively charged region, a central hydrophobic core and a polar carboxy-terminal domain. The protein contains a F/YRIP domain and four Asp boxes characteristic of bacterial and rodent Neu2 (Roggentin et al., 1993; Carillo et al., 1997). This region is highly conserved between the human and bacterial sialidases with sequence similarity from 32% to 38%.

The human Neu1 has three potential N-glycosylation sites at positions 185, 343 and 352. The positions of the active site residues are also conserved in bacterial, rodent and human Neu1. The conserved residues are Arg 37, 246 and 309 in Salmonella typhymurium, Arg 72, 274 and 341 in mice and Arg 78, 280 and 347 in humans (Crennell et al., 1993; Pshezhetsky et al., 1997; Igdoura et al., 1998). The predicted molecular mass of the Neu1 precursor is 45.4 kDa (Bonten et al., 1996) and after the cleavage of the signal peptide and glycosylation, the mature and active form of the protein has a mass of 48.3 kDa (Vinogradova et al., 1998).

1.2.3.4 Mutations in the *Neu1* gene

Since the identification of the *Neu1* cDNA (Bonten et al., 1996; Pshezhetsky et al. 1997; Milner et al., 1997), considerable progress has been made in the understanding of the molecular defects and biochemical mechanism of sialidosis. In two siblings with sialidosis type I, Bonten et al. (1996) identified a heterozygous

1258G→ú transversion that generated a premature TAG termination codon at amino acid 377 and caused a C-terminal truncation of 38 amino acids. In sialidosis type II patients Pshezhetsky et al., (1997) identified two missense mutations: a 779 T→ú (Phe260Tyr) transversion and a 1088 T→ú transition (Leu363Pro) in a cell line, GM01718A, obtained from a 2-month old infant with sialidosis type II. Another mutation was a frameshift caused by an ACTG duplication after nucleotide 7 (7insACTG) in the GM11604 cell line from a sialidosis type II patient. In another patient with sialidosis type II, Bonten et al. found a compound heterozygosity for a 401T→ú transversion (Leu91Arg) in one allele and the other allele contained a 1337delG deletion that caused a frameshift at amino acid 403 and a 69-aa extension of the protein. The extension produced a 53-kDa protein immunoprecipitated from the patient"s fibroblasts. In the SMJ mice with a deficiency of the Neu1 neuraminidase, Rottier et al. (1998) identified a 625C→ú (Leu209Ile) transversion, which accounts for the partial deficiency of Neu1.

1.2.3.5 Processing and lysosomal targeting of human Neu1

Several mechanisms have been proposed for sorting of the Neu1 precursor to the lysosome. Van der Spoel et al. (1998) compared the intracellular distribution of human Neu1 expressed in COS-1 cells transfected with *Neu1* cDNA alone or co-transfected with *Neu1* and human *CathA* cDNA, and suggested that Neu1 associates with CathA precursor shortly after synthesis and that this complex is targeted to the lysosome using a mannose-6-phosphate receptor-dependent pathway. In the absence of CathA, Neu1 is partially secreted and partially segregates to endosomal compartment (van der Spoel et al., 1998). In contrast, numerous data demonstrated that there are two pools of Neu1 in the lysosome, soluble and membrane associated. Both forms are absent in cultured cells of sialidosis patients and are encoded by the same gene (Miyagi et al., 1990, 1992, 1993; Verheijen et al, 1983). Furthermore, the activation of T lymphocytes is associated with several-fold increase of sialidase activity on the cell surface. The activation does not occur in T-cells obtained form SM/J or SM/B10 mouse strains with a mutation in Neu1 gene (Landolfi et al., 1985;

Naraparaju and Yamamoto, 1994). This fact also demonstrates that sialidases expressed on the cell surface and in the lysosome are the products of the same gene. Analysis of the deduced amino acid sequence of Neu1 (Pshezhetsky et al., 1997) revealed that C-terminal tetrapeptide, ^{412}YGTL^{415} has similarity to the internalization signals of several endocyted surface receptors and lysosomal membrane proteins. As described above the Tyr-X-X-θ (hydrophobic residue) internalization signal have been previously determined in cytoplasmic domains of several internalized membrane proteins, such as glucocerebrosidase LAMP-I, LAMP-II, lysosomal membrane glycoprotein (LGP-85), low density lipoprotein (LDL), transferrin, asyaloglycoprotein, polymeric immunoglobulin and cation-independent mannose-6-phosphate receptor, (Peters and Figura, 1994, Pearse et al., 2000; Hirst and Robinson, 1998).

1.3 Lysosomal storage diseases

In 1972, Hers introduced the concept of lysosomal storage diseases (LSD) to explain how the deficiency of a lysosomal enzyme, α-glucosidase, could be lethal in Pompe disease. The undegraded substrate would gradually accumulate within the lysosomes, increasing the size and number of organelles and thus leading to cellular death and eventually to the malfunctioning of the affected organ (Hers, 1972; Van Hoof, 1976). Later other disorders have been described caused by the molecular defects that resulted in deficiency of lysosomal enzymes affecting the enzyme'¯s synthesis, processing, routing, folding, maturation, activation, stability and oligomerization or complex formation. LSD may manifest as several clinical forms with different onset (infantile, juvenile or adult) or severity. Although clinical features generally vary between different disorders, the most common characteristics include hepatosplenomegaly, neuronal deterioration, skeletal complications, coarse faces, blindness and growth retardation.

At least 41 genetically distinct and biochemically related LSD caused by the absence of one or more lysosomal hydrolases have been described (reviewed in

Neufeld, 1991). Based on the clinical and biochemical manifestations of the disease, LSD are grouped into different classes: sphingolipidosis, mucopolysaccharidosis, mucolipidosis, glycoprotein storage diseases, lysosomal membrane transport disorders and others types. LSD are considered rare and each one typically affects fewer than 10,000 people in the world, although high prevalence values have been reported in some populations (Meikle et al., 1999) Most LSD are inherited in an autosomal recessive manner, with the exception of Fabry disease and mucopolysaccharidosis (MPS) type II, which show X-linked recessive inheritance.

The biggest group of lysosomal storage diseases is related to deficiencies of glycosidases involved in the catabolism of sugar chains of glycolipids, oligosaccharides and glycoproteins. This group includes single enzyme deficiencies as in Gaucher, Tay-Sachs and Sandhoff diseases, as well as the functional deficiencies of multiple enzymes caused by genetic mutations in sphingolipid activator proteins (saposins, G_{M2}-activator protein) which stimulate the activity of glycosidases against gangliosides and glycoshpingolipids.

Both Tay-Sachs and Sandhoff diseases are caused by a failure in the catabolism of G_{M2}-ganglioside. Protein products of three genes are required for this process: *HEXA* and *HEXB* genes encoding the αÁanβ¦Â subunits of hexosaminidase A and the gene encoding the G_{M2}-activator, a lipid-binding protein that presents the G_{M2} substrate to the enzyme. A defect in the *HEXA* gene causes Tay-Sachs disease, an autosomal recessive, progressive neurodegenerative disorder. Although less than 0.3 % of the general populations are carriers for the disease, the frequency is ten fold higher (3 %) in Ashkenazi Jews (Petersen et al., 1983). This disease is characterized by the developmental retardation, followed by paralysis, dementia and blindness and usually causes death at the age of 2 or 3 years. A defect in the *HEXB* gene leads to the clinically related Sandhoff disease.

Gaucher disease, another sphingolipidosis, is the most common lysosomal storage disease with an estimated carrier frequency of 4.6% among Ashkenazi Jews in Israel (Matoth et al., 1987). It is caused by deficiency of glucocerebrosidase, leading to the lysosomal accumulation of glucosylceramide.

Disorders caused by defects in the lysosomal membrane transporters include Nieman-Pick type C involving a cholesterol transport defect, sialic acid storage disease (sialic acid transport protein deficiency) and cystinosis (cystine transport protein deficiency).

A distinct group of disorders: galactosialidosis, β-galactosidosis and sialidosis involve the components of the 1.27 MDa lysosomal multi-enzyme complex of CathA, β-galactosidase and Neu1,

1.3.1 Galactosialidosis

Galactosialidosis (GS) is an autosomal recessive disease caused by a primary defect of protective protein/CathA which results in a combined secondary deficiency of β-galactosidase and Neu1 (Goldberg et al., 1971; Suzuki et al., 1988, Zhang and Callahan, 1996; D'Azzo et al., 1995). GS is characterized by typical lysosomal storage disease manifestations including coarse facies, macular cherry-red spots, mental retardation, corneal opacities, vertebral changes, bone marrow foam cells and vacuolated lymphocytes.

According to the severity and age of onset, GS can be classified into three phenotypically distinct forms: an early infantile, late infantile and juvenile/adult type. The early infantile onset manifest prenatally as non-immunologic hydrops fetalis, the excessive accumulation of serious fluid in the subcutaneous tissues and serous cavities of the fetus (Stone and Sidransky, 1999), or postnatally as ascites, massive edema, proteinuria, viceromegaly, skeletal dysplasia and early death from cardiac failure, kidney failure or airway obstruction (Wenger et al., 1978; Lowden and O'Brien, 1979). Some early-infantile GS patients have demonstrated cytopenias (abnormal decrease in number of cells in the bone marrow) (Olcay et al., 1998) and punctuate epiphyses of the femora, calcanei, and sacrum (Patel et al., 1999). The late-infantile onset is associated with hepatosplenomegaly, growth retardation, cardiac involvement and absence of relevant neurologic signs. The juvenile/adult onset, patients may survive to adulthood, often without mental retardation or viceromegaly, but with ataxia, myoclonus and angiokeratoma. About 70% of GS cases can be

classified in this group and the majority of reported patients are of Japanese origin (Suzuki et al., 1988; Takano et al., 1991). Parental consanguinity has been reported in half of the families.

First GS patients reported by Pinsky et al. (1974) and Loonen et al. (1974) had β-galactosidase deficiency but showed a clinical phenotype different from that of patients affected with β-galactosidosis (G_{M1}-gangliosidosis) with normal intelligence and late development of psychomotor deterioration. Galjaard et al. (1975) performed somatic cells hybridization studies, showing that these variants are caused by the defects in a gene other than β-galactosidase. Similarly, Wenger et al. (1978) found a combined deficiency of two lysosomal enzymes, β-galactosidase and Neu1. All reported patients belonged to the same complementation group, allowing designating the condition as a distinct disorder called galactosialidosis (GS) (Andria et al., 1981). For several years, primary molecular defect in GS was thought to be same as that in single Neu1 deficiency, sialidosis (Cantz et al., 1977). However, Hoogeveen et al. (1980, 1981) showed that hybridization and even co-culturing of fibroblasts from GS and sialidosis patients resulted in partial correction of Neu1 and β-galactosidase activity. These studies suggested a protein "corrective factor" exists in normal, sialidosis or G_{M1}-gangliosidosis cells, but absent in those of GS patients. Further studies showed a 10-fold enhanced cellular degradation of β-galactosidase in galactosialidosis fibroblasts (van Diggelen et al., 1982) that could be prevented by the addition to the cell medium of either a fraction containing the "corrective factor" or by the inhibition of lysosomal proteases (Suzuki, et al. 1988, d'Azzo et al, 1982).

CathA precursor is a 54 kDa protein which is processed in the lysosomes to the mature form containing two protein chains of 32 and 20 kDa. D'Azzo et al. (1982) showed that both the 32 kDa protein and its 54 kDa precursor are genetically absent in the cells of all GS patients and that addition of the 54 kDa precursor to these cells restores the normal level of β-galactosidase protein. Moreover, the 32-kDa subunit was identified as the "°corrective factor" missing in GS cells (D'Azzo et al., 1982).

It became possible to understand the molecular defects in GS only after the isolation and characterization of the gene encoding human CathA. The *CathA* gene

consists of 15 exons, spanning 7.5 kb on human chromosome 20 (20q13.1) (Galjart et al., 1988; Shimmoto et al., 1996). The protein is synthesized as a 542 amino acid precursor, glycosylated at Asn 117 in the ER, transported via the mannose-6-phosphate receptor (Morreau et al., 1992) to the lysosomes where it is activated by the proteolytic cleavage (Bonten et al., 1995).

A number of molecular lesions in the *CathA* gene in all subtypes of galactosialidosis have been identified. For instance, Zhou et al. (1996) found three mutations, V104M, L208P and G411S, in patients with early infantile GS. These mutations prevented the phosphorylation of the CathA precursor and thereby its transport to the lysosome. Examples of late infantile GS mutations include M378T, which generates a new Asn-linked glycosylation site, and Y221N, which decreases the stability of CathA in the lysosome (Zhou et al., 1996). A 2-nucleotide deletion, C517delTT, and an intronic mutation, IVS8+9C-G, which results in splice defect, generated frameshifts and a protein truncation (Richard et al., 1998). Among Japanese patients with juvenile/adult onset the most common mutations are a Y249N change (Fukuhara et al., 1992), and an IVS7, A-G, +3, EX7DEL mutation, resulting in skipping of exon 7 in the mRNA (Shimmoto et al., 1996).

Because the CathA protein has about 30% amino acid sequence identity with the yeast carboxypeptidase Y and the wheat carboxypeptidase-II (Galjart et al., 1988, Elsliger and Potier, 1994), the X-ray atomic coordinates of the wheat enzyme were used to build the CathA structure model (Elsliger and Potier, 1994). Later, the X-ray structure of the CathA precursor expressed in a baculovirus system was determined with a 2.2-2.4 resolution (Rudenko et al., 1995). The structure is similar to those of plant and yeast serine carboxypeptidases, showing that CathA belongs to a so-called αÁ-hydrolase family (Remington, 1993). The protein is composed of a cap domain and a core domain. The cap domain consists of three α-helixes and three-stranded mixed -sheet. The core domain consists of a central ten-stranded -sheet which is flanked by ten α-helixes and two small -strands on both sides. The catalytic triad in the active site is formed by the Ser^{150}, His^{429} and Asp^{372} residues. At acidic pH,

the enzyme forms 95-98 kDa homodimers with known X-ray structure (Rudenko et al., 1995). The pI for the human enzyme is 5.4.

The resolution of the X-ray structure of CathA precursor allowed a comprehensive analysis of structural changes induced by the mutations in CathA molecules (Rudenko et al., 1998). The analysis revealed a correlation between the effects of mutation on the protein structure and the clinical phenotype of the affected patients. None of the mutations occurred in the active site or at the protein surface. Among 11 amino acid substitutions modeled, 9 found in patients affected with severe early or late infantile type of GS (Q21R, S23Y, W37R, S62L, V104M, L208P, Y367C, M378T, and G411S) were located in the central core domain of CathA. These substitutions introduced unbalanced charged groups, hydrogen bonds or bulkier side chains to the protein core; or created cavities in protein interiors and interfaces. All these changes would dramatically change the folding of mutant CathA, resulting in impaired sorting and rapid degradation. In contrast, the other two mutations (F412V and Y221N) associated with a more moderate clinical effect were located in the α-helical cap domain of the enzyme and predicted to have a milder effect on protein structure. For several mutations (Q21R, W37R, S62L, Y221N, Y367C and F412V), homologous modeling of CathA structure (Elsliger and Potier, 1994) also made similar predictions.

1.3.2 Sialidosis

Sialidosis (SL) is a very rare inherited metabolic disorder characterized by a single Neu1 deficiency leading to the abnormal accumulation of complex carbohydrates (mucopolysaccharides) and mucolipids in many tissues of the body. Previously known as Mucolipidosis I (Cantz et al., 1977), the cherry-red spot myclonus syndrome (O'Brien et al., 196), the Goldberg syndrome (Thomas et al., 1978) and nephrosialidosis (Maroteaux et al., 1978) SL belongs to a subgroup of lysosomal diseases known as mucolipidoses. Lowden and O'Brien (1979) provided the nosology, sialidosis, and its classification into two phenotypically distinct subtypes: sialidosis type I and sialidosis type II, based on dysmorphic features,

severity and age of onset. **Sialidosis type I** (Goldberg syndrome or non-dysmorphic type) is a mild and late-onset form characterized by bilateral macular cherry-red spots, debilitating myoclonus (sudden involuntary muscle contractions) and progressive visual impairment (Durand et al., 1977; Soggs et al., 1979; Rapin et al., 1978; O'Brien, 1979; Federico et al., 1980). These symptoms usually do not appear until the second decade of life. O'Brien and Warner (1980) noted that sialidosis type I was more frequent in Italians. **Sialidosis type II** (mucolipidosis I, lipomucopolysaccharidosis or dysmorphic type) is a severe and infantile onset form associated with skeletal dysphasia, Hurleroid features (mildly coarse facial features), dysostosis multiplex (bone malformations), mental retardation, hepatosplenomegaly and death in the first decade of life (Kelly et al, 1977; Winter et al., 1980; Oohira et al., 1985). Earlier reports indicated that most type II patients were of Japanese origin (O'Brien and Warner, 1980). In a recent study, Nishiyama et al. (1997) reported a cerebral blood flow and glucose metabolism decrease in the occipital lobe region of Japanese patients with the adult onset sialidosis. Other symptoms may include abdominal swelling; loss of muscle mass (atrophy); irregular, involuntary spastic muscle movements (choreoathetosis); lack of muscle tone (hypotonia); the protrusion of a portion of the intestines through an abnormal opening in the muscular wall of the abdomen (inguinal hernia) (Provenzale et al., 1995) **Nephrosialidosis** (congenital sialidosis), a phenotypic variant of sialidosis type II, presents type II symptoms as well as severe hydrops fetalis, ascites and proteinuria. Death usually results from edema, dysproteinuria and bleeding (Maroteaux et al., 1978; Tylki-Szymanska et al. 1996). Besides the above clinical features, Buchholz et al. reported severe dilated coronary arteries, excessive retinal tortuosity and an erythematous, macular rash in an infant with congenital sialidosis.

Sialidosis is a very rare lysosomal storage disorder with insignificant prevalence in a selected population. Since Tipton et al. noted at least 11 cases in 1978, there have been no published reports on the global incidence of sialidosis. However, according to the Mucopolysaccharadosis (MPS) society, sialidosis occurrence is between 1 in 250,000 and 1 in 4.2 million (www.mpssociety.ca/diseases).

Various studies based on geographic and/or ethnic distribution of lysosomal storage diseases have been conducted. In the Netherlands, only 49 cases of mucolipidoses and oligosaccharidoses were diagnosed between 1970 and 1996, with a combined birth prevalence is 1.0 per 100,000 live births (Poorthuis et al., 1999). In Australia, 27 different lysosomal storage diseases were diagnosed in 545 patients and a prevalence ranging from 1 in 57,000 live births for Gaucher disease to 1 in 4.2 million live births for sialidosis was reported (Meikle et al., 1999). The overall incidence for all types of LSD in this study was approximately 1 in 7,700 live births. Since the Australian population has mainly a British ancestry, with a minor contribution from other European countries and Asia, Meikle et al. believed their results could be extrapolated to white non-Hispanic populations in the United States, Canada and the United Kingdom. At least fifteen cases of sialidosis type II have been reported in Japan (Nishiyama et al., 1997; Naganawa et al., 2000).

1.4 Biological role of Neu1 in the immune response

As discussed above, the role of Neu1 in the intralysosomal catabolism of sialilated glycolipids and glycoproteins has been well-established. Several studies have shown that the Neu1-encoded sialidase, in addition to its role in intralysosomal catabolism of sialylated glycoconjugates, is also involved in cellular signaling during the immune response. Endogenous sialidase activity increases in cells of the immune system following cell activation (Chen et al., 1997 and Katoh et al., 1999). The enhanced sialidase activity and consequent desialylation of surface glycoconjugates in activated cells induced production of interleukin-4 by lymphocytes. In particular, T cells require Neu1 for both early production of IL-4 and the IL-4 priming of conventional T cells to become active IL-4 producers (Chen et al., 1997). During the activation of T cells, Neu1 is expressed on the plasma membrane where it participates in desialylation of surface antigen-presenting molecules such as myosin heavy chain class I, required to render T cells responsive to APCs (Landolfi et al., 1986), and G_{M3}-ganglioside, which modulates Ca^{2+} immobilization and regulates IL-4 production (Chen et al., 2000). In addition, Neu1 of T lymphocytes converts the

group specific component or Gc protein into a factor necessary for the inflammation-primed activation of macrophages (Naraparaju et al., 1994). T-cells derived from SM/J or B10.SM strains of mice, partially deficient in Neu1 (Carrillo et al., 1997), fail to convert Gc and synthesize IL-4, and B cells of these mice cannot produce IgG_1 and IgE after immunization with pertussis toxin. Since binding of Neu1 to CathA is required for activation it is possible to suggest that these two enzymes remain associated on the plasma membrane. Therefore, Neu1 may interact with CathA precursor in the Golgi and stay associated with it on the root to the plasma membrane. In accordance with this hypothesis recent studies demonstrated the presence of enzymatically nonactive CathA precursor on the plasma membrane (Hinek et al., 1996).

Lukong et al., (2001) have shown that in the activated T-cells, Neu1-encoded sialidase is increased on the cell surface in good agreement with the data of Chen and Landolfi. Lukong et al., further investigated the mechanism by which Neu1 reaches the cell surface and showed that it contains the internalization signal found in lysosomal membrane proteins targeted to endosomes via clathrin-coated pits. The signal consists of a C-terminal tetrapeptide ^{412}YGTL^{415}, with Tyr^{412} and Leu^{415} essential for the endocytosis of the enzyme. In turn, the redistribution of Neu1 from lysosomes to the cell surface of activated lymphocytes was accompanied by increased reactivity of the enzyme towards anti-phosphotyrosine antibodies. Lukong et al., suggested that a specific mechanism regulates sorting and retention of newly synthesized Neu1 on the surface of activated T cells. They proposed that the interaction of Neu1 with adaptor complexes and targeting to the lysosome can be blocked by phosphorylation of the essential tyrosine in the internalization motif. Such a mechanism has been recently described for cytotoxic T lymphocyte-associated antigen (CTLA-4), which is transiently expressed on the surface of activated T cells and is involved in their down-regulation where it may influence immune function (van der Horst et al., 1989; Lukong et al., 2000). In activated T cells, phosphorylation of the essential Tyr^{165} residue by the T-lymphocyte associated tyrosine Src family and

Jak2 kinases prevents its interaction with the ¦ Ì2 ubunit of AP2 and results in its expression on the cell surface (Bradshaw et al., 1999 and Chikuma et al., 2000).

Several lines of evidence also suggest that Neu1 plays a pivotal role in cellular signaling during monocytes differentiation and activation. For example, activation of monocytes caused significant induction of sialidase activity which enhanced binding of CD44 on the surface of monocytes to hyaluronic acid, a component of the extracellular matrix (Gee et al., 2003). In this study, they showed that LPS-induced CD44-mediated HA (CD44-HA) binding in monocytes is regulated by endogenously produced tumor necrosis factor (TNF)-α and IL-10. Furthermore, p38 mitogen- activated protein kinase (MAPK) activation was required for LPS- and TNF-α-induced, but not for the IL-10-induced CD44-HA-binding in normal monocytes. It suggested that Neu1 activation may be required for the acquisition of the HA-binding form of CD44 in LPS- and TNF-stimulated monocytic cells.

In previous studies, LPS stimulation increased the Neu1 activity in monocytes, but it is not known whether it was due to the gene induction or post-transcriptional activation (Katoh et al., 1999). The *Neu1* induction is not only specific for T-cells and monocytes. The induction of the *Neu1* gene by IL-1 was also observed in human blood neutrophils, where it was proposed to be involved in tissue recruitment (Cross et al., 2002). IL-1 is known to have a specific stimulatory effect on neutrophil recruitment (Lin et al., 2002), but whether or not the Neu1 induction plays a role in the IL-1-induced neutrophil recruitment remains to be elucidated.

In the SM/J mice, partial Neu1 deficiency is specifically associated with the suppressed expression of Th2, but not Th1, cytokines (Chen et al., 1997). However, in cultured human T-cells, overexpression of Neu1 resulted in enhanced production of both Th1 and Th2 cytokines (Wang et al., 2004). There are two possible explanations for this apparent discrepancy. One possibility relates to species difference, as suggested by the difference of Neu1 regulation in mice and humans (Landolfi et al., 1986 and Lukong et al., 2001). Alternatively, and more plausibly, the specific association of Neu1 with Th2 cytokine expression may happen *in vivo* only. In fact, it is reported that in mouse T-cells and DCs treatment with a viral Neu1

resulted in enhanced production of multiple cytokines, including both Th2 and Th1 (Oh et al., 2000). Taken together these results suggest that Neu1 may indeed be involved in the expression of Th1 and Th2 cytokines in both humans and mice.

Previously identified (Monti et al., 2000) plasma membrane-associated sialidase Neu3 promotes cell adhesion to laminins, integrin-mediated signaling to ERK and subsequent activation of cell proliferation, but attenuates adhesion to fibronectin and the related signaling (Kato et al., 2006). There is also evidence that Neu3 overexpression may contribute to sustained ERK activation through Shc and FAK signaling, when cells are attached to laminin-5, leading to adhesion-dependent cell proliferation. Neu3 overexpression in T-cells resulted in enhanced production of several cytokines of both Th1 and Th2 types, but not of IL-4. These results suggest that gangliosides blocked Th1 cytokine expression without affecting IL-4 (Wang et al., 2004). On the other hand, the differential effects of Neu1 and Neu3 on IL-4 expression may be due to the fact that the two sialidases have different substrates on the cell surface.

Neu1 and Neu3 may regulate cytokine genes expression by processing certain gangliosides on the cell surface. Gangliosides are known to be involved in a variety of biological functions in most (if not all) cells, including cytokine gene regulation in T-cells (Kanda et al., 2001; Irani et al., 1996) and antigen-presenting cells (Shen et al., 2002). It is possible that Neu1 and Neu3 may hydrolyse different gangliosides *in vivo*, which may explain why they have differential effects on cytokine genes expression. Neu1 and Neu3 may also activate intracellular signaling, which leads to cytokine production by desialylating cell surface glycoproteins, such as TCR, CD4, CD8, CD43, CD45, IFN-γÃrecept α, andigands for siglecs (Pappu et al., 2004 and Ikehara et al., 2004). Future experiments using lymphocytes from individuals affected with sialidosis (primary Neu1 deficiency) and galactosialidosis (secondary Neu1 deficiency) may help to establish the specific substrates for Neu1 and Neu3 and define the contribution of each enzyme to the production of IFN-γÃIn addition to influencing the production of IFN-γÃ Neu and/or Neu3 may play a role in some of the other immune functions of human lymphocytes. This prospect raises the

possibility that inhibition of the activity of cellular sialidases with anti-sialidase antibodies or pharmacologic inhibitors may have a therapeutic value in treating inflammation and infection.

1.5 Research hypotheses and objectives

The previous studies from our lab and published data summarized above showed that Neu1, in addition to its role in intralysosomal catabolism of sialylated glycoconjugates, is also involved in cellular signaling during the immune response participating in the production of cytokines by T cells. There is also indirect evidence that Neu1 may also play an important role in other types of immune cells, in particular in monocytes, macrophages and dendritic cells. These cells play a central role in innate and adaptive immune responses and their activation is central to the outcome of virtually every infectious disease. In addition a failure to properly regulate macrophage function during infection is often itself a major cause of disease. For example, chronic infections, such as tuberculosis, are often characterized by the inability of macrophages to eliminate persistent infections. On the other hand, in septic shock, caused by acute infections, hyperactivated macrophages initiate cascades of immune deregulation often resulting in death. Therefore understanding of the signaling pathways in macrophages holds great promise for future development of therapeutic targets for a plethora of inflammatory diseases, but in contrast to the relatively well-understood pathways in innate immune responses, the signaling pathways that are necessary to counter and temper aggressive macrophages are poorly characterized.

Our major hypothesis is that the lysosomal sialidase Neu1 and /or CathA, which forms with Neu1 the lysosomal multi-enzyme complex and activates it in the lysosome play a crucial role in signaling, during the differentiation and activation of macrophages through transcriptional induction and mobilization to the cell surface in stimulated cells. In order to test this hypothesis, the specific aims are based on the analysis of cell lines and cells from two mouse models generated in our laboratory. The mouse models are: the *CathA*^{S190A} point mutation with single CathA deficiency,

and the *CathA*$^{S190A\text{-}Neo}$ hypomorph with a double CathA/Neu1 deficiency. These models are keys to our studies because they link biochemical deficits in the protein components of the lysosomal multienzyme complex to end-stage phenotypes only amenable through the functional organism.

Our specific aims are:

1. To study the expression of Neu1 and other sialidases during the activation of blood monocytes and monocytic cell lines.
2. To study the regulation of *Neu1* gene transcription in monocytes and macrophages.
3. To study the targeting of Neu1 and CathA in monocytes and macrophages.
4. To study the biological role of Neu1 in cell signaling during the maturation and activation of immune cells using siRNA and the cells of transgenic mice with a single CathA and double CathA/Neu1 deficiency.

CHAPTER 2: DIFFERENTIAL EXPRESSION OF ENDOGENOUS SIALIDASES OF HUMAN MONOCYTES DURING CELLULAR DIFFERENTIATION INTO MACROPHAGES

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DIFFERENTIAL EXPRESSION OF ENDOGENOUS SIALIDASES OF HUMAN MONOCYTES DURING CELLULAR DIFFERENTIATION INTO MACROPHAGES

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ABSTRACT

Sialidases are enzymes that influence cellular activity by removing terminal sialic acid from glycolipids and glycoproteins. Four genetically distinct sialidases have been identified in mammalian cells. In this study, we demonstrate that three of these sialidases, lysosomal Neu1 and Neu4 and plasma membrane-associated Neu3, are expressed in human monocytes. When measured using the artificial substrate 2'-(4 methylumbelliferyl)-a-d-Nacetylneuraminic acid (4-MU-NANA), sialidase activity of monocytes increased up to 14-fold per milligram of total protein after cells had differentiated into macrophages. In these same cells, the specific activity of other cellular proteins (e.g. β-galactosidase, cathepsin A and alkaline phosphatase) increased only two- to fourfold during differentiation of monocytes. Sialidase activity measured with 4-MU-NANA resulted from increased expression of Neu1, as removal of Neu1 from the cell lysate by immunoprecipitation eliminated more than 99% of detectable sialidase activity.

When exogenous mixed bovine gangliosides were used as substrates, there was a twofold increase in sialidase activity per milligram of total protein in monocyte-derived macrophages in comparison to monocytes. The increased activity measured with mixed gangliosides was not affected by removal of Neu1, suggesting that the expression of a sialidase other than Neu1 was present in macrophages. The amount of *Neu1* and *Neu3* RNAs detected by real time RT-PCR increased as monocytes differentiated into macrophages, whereas the amount of *Neu4* RNA decreased. No RNA encoding the cytosolic sialidase (Neu2) was detected in monocytes or macrophages. Western blot analysis using specific antibodies showed that the amount of Neu1 and Neu3 proteins increased during monocyte differentiation. Thus, the differentiation of monocytes into macrophages is associated with regulation of the expression of at least three distinct cellular sialidases, with specific up-regulation of the enzyme activity of only Neu1.

INTRODUCTION

Sialic acid is present on glycoproteins and glycolipids that are widely distributed throughout nature. Removal of sialic acid from these glycoconjugates on the surface of mammalian cells changes the functional capacity of the cells [1–8]. Sialidases comprise a family of enzymes that remove terminal sialyl residues from glycoconjugates. Four genetically distinct forms of mammalian sialidase have been characterized, each with a predominant cellular localization (lysosomal, cytosolic or plasma membrane-associated) and substrate specificity [9–17]. Lysosomal sialidase (Neu1) has a catabolic role in desialylating glycoproteins and glycolipids in lysosomes [18], but is also present on the surface of activated T cells [19], where it may influence immune function [2,20]. Plasma membrane sialidase (Neu3) localizes on the cell surface [13, 14] and, by preferentially desialylating gangliosides, is believed to have a regulatory role in cellular activation, differentiation and transformation [4,21–23]. The cytosolic sialidase (Neu2) can desialylate both glycoproteins and gangliosides [12], but its function remains to be determined. The function of the recently characterized Neu4 sialidase also has not been established. Neu4 sialidase is expressed in a wide range of cell types [15–17], has broad substrate specificity, and is localized in lysosomes [17].

Endogenous sialidase activity increases in cells of the immune system following cell activation [2,5,6,20,24–27]. The enhanced sialidase activity and consequent desialylation of surface glycoconjugates in activated cells induced production of interleukin-4 by lymphocytes [2], enhanced binding of CD44 on the surface of monocytes to hyaluronic acid, a component of the extracellular matrix [5,27], and promoted the transendothelial migration of polymorphonuclear leukocytes (PMNs) [7]. In activated lymphocytes [2,20] and PMNs [7], the effect on cells was attributed to the activity of Neu1 sialidase, some of which was translocated from lysosomes to the cell surface [7,19]. The role of the other forms of sialidase in the activation of these cells has not been determined.

Circulating peripheral blood monocytes play a key role in potentiating diverse immune activities and can differentiate into either macrophages or dendritic cells by

exposure to specific stimuli [28]. The function of monocytes changes from antigen recognition and processing to antigen presentation in macrophages and dendritic cells. We have previously shown that desialylation of glycoconjugates on the surface of freshly isolated monocytes using an exogenous bacterial neuraminidase activated the extracellular signal-related kinase 1 / 2 (ERK 1 / 2), enhanced the production of specific cytokines, and promoted the responsiveness of monocytes to bacterial lipopolysaccharide [29]. In this paper, we demonstrate that endogenous sialidase activity of freshly isolated human monocytes is up-regulated as they differentiate into macrophages. We show that (a) Neu1 and Neu3 are present in both monocytes and macrophages, and that the specific activity of only Neu1 is up-regulated in comparison to other lysosomal proteins during differentiation; (b) Neu4 is also expressed in monocytes as evidenced by the presence of *Neu4* RNA, but that the amount of this RNA declines during monocyte differentiation; and (c) Neu2 is not detected at the RNA level in either monocytes or macrophages.

EXPERIMENTAL PROCEDURES

Isolation of peripheral blood mononuclear cells and purification of monocytes

Peripheral blood mononuclear cells were isolated by leukophoresis of blood from HIV-1 and hepatitis B and C seronegative donors followed by centrifugation over Ficoll-Paque Plus (Amersham Biosciences, Uppsala, Sweden) gradients using standard procedures. Monocytes were purified from peripheral blood mononuclear cells by an additional centrifugation over Percoll (Amersham Biosciences, Uppsala, Sweden) gradients and then by negative selection using StemSep separation columns (Stem Cell Technologies, Vancouver, BC, Canada) as per the manufacturer's suggested protocol. The purity of monocytes exceeded 95% as determined by flow cytometry after staining cells with phycoerythrin (PE)-, allophycocyanin (APC)-, or fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies to CD3, CD14, CD19, CD206 and isotypic control IgGs (all mAbs from BD PharMingen, San Diego, CA, USA). Briefly, $1x10^6$ cells were resuspended in 0.5 mL of a solution containing NaCl / Pi pH 7.4, 2% human serum AB and anti-CD32 Fc receptor Abs (1.5 μg)

(Stem Cell Technologies) and incubated at 4°C for 15 min to minimize non-specific binding of reagents. Cells were then stained at 4°C for 30 min with the fluorochrome-conjugated monoclonal antibodies, washed with 2 mL of NaCl / Pi and fixed with 1.0% (v / v) paraformaldehyde. Cells were analyzed using a Becton-Dickinson FACScaliber (Mountain View, CA, USA) and data were analyzed using FLOWJO data analysis software. The viability of monocytes was greater than 97% as determined by trypan blue dye exclusion.

Culture conditions for purified monocytes

To obtain monocyte-derived macrophages, purified monocytes were suspended at $2x10^6$ cells/mL in RPMI medium 1640 (Gibco, Grand Island, NY, USA) containing 10% heat-inactivated human AB serum (Gemini Bioproducts, Calabasas, CA, USA) and recombinant human macrophage colony stimulating factor (rhM-CSF; R&D Systems, Inc., Minneapolis, MN, USA) at 10 ng/mL and were maintained at $2.5x10^6$ cells per well in six-well tissue culture plates (Costar, Corning Inc., Corning, NY, USA) at 37°C in a 5% (v / v) humidified CO_2 incubator. At the indicated times, nonadherent cells were removed by two washes with NaCl / Pi pH 7.4 and the adherent, differentiating macrophages (larger and more granular than monocytes as seen by light microscopy) were harvested in NaCl / Pi pH 7.4 by gentle scraping with a polyethylene cell scraper (Nalge Nunc International, Rochester, NY, USA). The harvested cells were confirmed to have characteristic macrophage cell surface phenotypic markers (CD14+, CD206+) by flow cytometry that was performed as described above.

Measurement of sialidase activities

Cells were collected on the indicated days and $2x10^6$ monocytes (day 0) or 5 $x10^5$ cells on days 3 and 7 were suspended in 0.20 mL of a solution containing 0.5% (v / v) Triton X-100, 0.05 M sodium acetate pH 4.4, and 0.125 mM 4-MU-NANA (Sigma-Aldrich, St. Louis, MO, USA) and incubated at 37°C for 1 h. The reaction was terminated by the addition of 1.0 mL of a solution containing 0.133 M glycine,

0.06 M NaCl and 0.083 M Na_2CO_3 pH 10.7. Liberated 4-methylumbelliferone was measured with a Victor2 1420 spectrofluorometer (Wallac, Turku, Finland) with excitation at 355 nm and emission at 460 nm. The amount of 4-methylumbelliferone that was liberated from 4-MU-NANA during the 1 h reaction was determined by comparison to a standard curve of increasing amounts of 4-methylumbelliferone (Sigma-Aldrich). In this assay, 1 nmol of liberated 4-methylumbelliferone signified the release of 1 nmol of sialic acid, and a unit of sialidase activity was defined as the amount of enzyme that released 1 nmol of sialic acid per hour at 37°C. Protein concentration was measured by the Bradford method using a protein assay kit (Bio-Rad, Hercules, CA, USA) and the amount of activity measured in each sample was corrected based on protein concentration to represent activity per milligram of protein as seen in Fig. 1.

Sialidase activity was also determined against mixed bovine brain gangliosides (Calbiochem, La Jolla, CA, USA) and in the absence of exogenous substrate (i.e. where activity reflects the release of sialic acid from endogenous cellular sialylconjugates). In these assays, cells were collected on the indicated days and $2x10^6$ cells were suspended in 0.20 mL of a solution containing 0.1% (v / v) Triton X-100, 0.05 M sodium acetate pH 4.4, 0.1% (w / v) BSA (Pentex bovine albumin fraction V, Miles Inc., Kankakee, IL, USA) and 0.250 mM mixed bovine brain gangliosides. Alternatively, the gangliosides were omitted from the reaction mixture such that any detected free sialic acid would be that released from cellular sialylconjugates. After a 60 min incubation at 37°C, the reaction mixture was microfuged to remove cellular debris and 0.02 mL of each supernatant was analyzed for sialic acid content using a Dionex DX600 chromatography system (Dionex Corporation, Sunnyvale, CA, USA) equipped with an electrochemical detector (ED50, Dionex Corporation), as described previously [7]. Material from each 0.02 mL sample was injected into a CarboPac-PA1 column (4 x 250 mm) in the presence of 0.1 M NaOH, and sialic acid was eluted using a gradient of 5–20% (w / v) 1 M sodium acetate in 0.1 M NaOH over 15 min at a rate of 1 mL / min. Under this condition, sialic acid was eluted at 8.7 min and was quantified by integration of the

peak area using a standard solution of sialic acid as the reference. One unit of sialidase activity was defined as the amount of enzyme that liberated 1 nmol of sialic acid per hour at 37°C. The amount of activity measured in each sample was corrected based on protein concentration to represent activity per milligram of protein as seen in Fig. 1.

Quantitation of other lysosomal and cellular proteins

Freshly isolated monocytes and macrophages after 7 days in culture were collected and homogenized in H_2O by sonication. Hexosaminidase and β-galactosidase activity were measured separately by incubating 5μg of cell homogenate in 0.1mL of a solution containing 40 mM sodium acetate pH 4.6 and either 1.25 mM 4-methylumbelliferyl-2-acetamido-2-deoxy-β-d-glucopyranoside or 1.5 mM 4-methylumbelliferyl-β-d-galactoside as previously described [51,52]. After incubation at 37°C for 15 or 30 min, the reactions were terminated with 1.9 mL of 0.4 M glycine buffer pH 10.4 and the amount of fluorescence of the liberated 4-methylumbelliferone was measured with a Shimadzu RF-5301 spectrofluorometer. Alkaline phosphatase, glutamate dehydrogenase and cathepsin A activities in 5 μg of cell homogenate were measured as described elsewhere [34,53,54]. The amount of lysosome-associated membrane protein-2 (LAMP-2) in monocytes and macrophages was determined by separating cellular proteins by SDS / PAGE, electrotransferring them to polyvinyldifluoride membranes, and reacting the proteins that were transferred to the blots with monoclonal antihuman LAMP-2 antibodies (Washington Biotechnology Inc., Baltimore, MD, USA). Antibody-bound LAMP-2 was detected using the BM chemiluminescence kit (Roche Diagnostics, Mannheim, Germany) in accordance with the manufacturer's protocol.

Immunoprecipitation of Neu1 multienzyme complex

Neu1 exists in a multienzyme complex with β-d-galactosidase and cathepsin A [18,31–34] and can be immunoprecipitated selectively from cell lysates using anti-cathepsin A antibodies [34]. Neither Neu2 nor Neu3 form oligomeric structures when

purified from tissues [55,56]. In addition, when COS-7 cells were transfected with plasmids that expressed Neu3 or Neu4 and cell lysates were reacted with anti-cathepsin immune serum, neither Neu3 nor Neu4 sialidases were immunoprecipitated [K. Landry, unpublished results]. Freshly isolated monocytes or monocyte-derived macrophages (10^6 cells) were homogenized in 0.55 mL of a solution containing 100 mM NaCl, 0.5% (w / v) of sodium desoxycholate, and 50 mM sodium phosphate buffer, pH 6.0. After centrifugation of the homogenate at 12000 g for 10 min, 0.20 mL of the supernatant was mixed with 0.10 mL of a solution containing 10 mg/ mL BSA, 100 mM NaCl, and 50 mM sodium phosphate buffer, pH 6.0 with 5 μg of rabbit anti-human cathepsin A immune serum or preimmune serum and incubated at 4°C for 1 h as described elsewhere [34]. The pellet from 0.300 mL of Pansorbin Cells (Calbiochem, La Jolla, CA, USA) was added to the reaction mixture after the 1 h incubation and the sample was incubated for an additional 1 h at 4°C with constant shaking. The immune complexes were removed from the supernatant by centrifugation at 13000 g for 10 min. The supernatants were assayed for β-galactosidase (GAL), β-hexosaminidase (HEX), and sialidase activities as described above.

Isolation of RNA and real time RT-PCR

Monocytes and monocyte-derived macrophages were harvested as previously described and total RNA was isolated using an RNeasy mini kit (Qiagen, Valencia, CA, USA) following the protocol suggested by the manufacturer. The RNA preparation was treated with DNase I (Invitrogen, Carlsbad, CA, USA) at 37°C for 30 min to remove contaminating DNA. DNase was then removed by binding to Blue Sorb DNase affinity slurry (Clonogene, St. Petersburg, Russia).

Semi-quantitative real-time RT-PCR was performed using a QuantiTect SYBR green RT-PCR Kit (Qiagen, Valencia, CA, USA) with an ABI Sequence Detection System (ABI PRISM 5700) to detect gene expression of Neu1 (GenBank accession NM_000434), Neu2 (GenBank Accession NM_005383), Neu3 (GenBank accession AB008185), and Neu4 (GenBank accession NM_080741) using RNAs

generated as described above. Gene expression of 18S rRNA (GenBank accession X03205) was also measured as an internal control. The following primers were selected using Primer Express v1.0 (Applied Biosystems, Foster City, CA, USA) or DNAsis Max (Hitachi, Japan) software and were synthesized by Qiagen (Germantown, MD, USA): *Neu1* (forward; nt 1047–1066) 5'-TGTGACCTTCGACCCTGAGC-3' and (reverse; nt 1151–1170) 3'-CTCACTTGGACTGGGACGCT-5' yielding a 123 base product; *Neu2* (forward; nt 458–477) 5'-AGTGGTCCACCTTTGCAGTG-3' and (reverse; nt 581–600) 3'-GGAAGACGAAGGAGTCGGTA-5' yielding a 142 base product; *Neu3* (forward; nt 844–864) 5'-AATGTGAAGTGGCAGAGGTGA-3' and (reverse; nt 971–991) 3'-GGACTCAGCTGTCGAGACACT-5' yielding a 147 base product; *Neu4* (forward; nt 1002–1020) 5'-TGCTGGTACCCGCCTACAC-3' and (reverse; nt 1085–1104) 3'-AAGATGTCGCTACTGGTGCC-5' yielding a 103 base product; and 18S rRNA (forward: nt 1279–1298) 5'-CGGACAGGATTGACAGATTG-3' and (reverse; nt 1378–1397) 3'-TTGCTTGCTCTGAGACCGTA-5' yielding a 119 base product. Ten nanograms (10 ng) of total RNA was added to a 25 μL final reaction mixture containing 0.5 μM of each primer pair, 1 x QuantiTect SYBR-green RT-PCR Master Mix and 0.25 μL of QuantiTect RT Mix. To synthesize cDNA, reverse transcription was performed at 50°C for 30 min. Following a 15 min hot start at 95°C, DNA amplification was allowed to proceed for 40 cycles (15 s at 95°C, 30 s at 57 °C and 30 s at 72°C). All reactions were run in triplicate. Semi-quantitative analysis was based on the cycle number (CT) at which the SYBR-green fluorescent signal crossed a threshold in the log-linear range of RT-PCR, indicating the relative amount of starting template in each sample. The fold change in expression of *Neu1*, *Neu3*, and *Neu4* RNAs in macrophages compared to monocytes was normalized to the expression of 18S rRNA and was calculated by equation $2^{-\Delta\Delta CT}$ where $\Delta\Delta C_T = (C_{T\ Neu1,2\ or\ 3} - C_{T\ 18S\ rRNA})_{macrophages} - (C_{T\ Neu1,2\ or\ 3} - C_{T\ 18S\ rRNA})$ monocytes. The accuracy of each reaction was monitored by analysis of melting curves and product size on gel electrophoresis.

Western blot analysis of cellular proteins

Monocytes and macrophages were collected at the indicated times and proteins from 2 x10^6 cells were solubilized in 0.1 mL of a solution containing 50 mM Tris / HCl pH 7.4, 100 mM NaCl, 0.5% (v / v) Triton X-100, 0.5% (w / v) sodium desoxycholate, 0.1% (w / v) SDS and protease inhibitors (1 : 250 dilution of protease inhibitor cocktail from Sigma-Aldrich). Protein concentration was measured by the Bradford method using a Bio-Rad protein assay kit (Bio-Rad). Proteins (5 µg) from each cell lysate were resolved by electrophoresis on a 10% SDS / polyacrylamide gel using Tris / glycine /SDS running buffer (gel and running buffer from Invitrogen, Carlsbad, CA, USA), electrotransferred by a semi-wet method to a Sequi-Blot polyvinyldifluoride membrane (Bio-Rad) and probed with polyclonal rabbit antibodies to either Neu1 or Neu3 at 0.5 µg / mL The polyclonal anti-Neu1 Igs were generated by immunizing rabbits with recombinant human Neu1 sialidase and were characterized as described elsewhere [38]. Rabbit polyclonal anti-Neu3 Igs were generated by immunizing rabbits with a synthetic peptide corresponding to amino acids 109–128 of the human Neu3 sialidase and were affinity-purified using the immunogen that was coupled to a column. These anti-Neu3 Igs detected a single 47 kDa band in COS-7 cells that were transfected with the Neu3 gene. The respective blots were incubated with a 1 : 10 000 dilution of goat HRP-conjugated anti-rabbit IgGs (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), developed using an ECL chemiluminescence substrate kit (Amersham Biosciences, Piscataway, NJ, USA), and exposed to Kodak X-ray film.

RESULTS

Differentiation of monocytes into macrophages results in increased expression of endogenous sialidase(s)

To determine whether differentiation of monocytes into monocyte-derived macrophages is associated with changes in the level of endogenous sialidase activity, monocytes were purified from the peripheral blood of human donors and maintained in culture conditions that promoted differentiation into macrophages. The amount of

sialidase activity in freshly isolated monocytes (CD14+, CD206–) and in monocyte-derived macrophages (CD14+, CD206+) after 3 and 7 days in culture was determined using the exogenous sialidase substrates 2'-(4-methylumbelliferyl)-α-d-N-acetylneuraminic acid (4-MU-NANA) and mixed bovine gangliosides. These substrates are utilized with different efficiencies in vitro by the four genetically distinct mammalian sialidases [10,13,14,30]. Sialidase activity of cells was also evaluated in the absence of exogenous substrates to determine whether any of the cellular sialidases was able to desialylate endogenous sialylconjugates under the conditions that were used. Sialidase activity from solubilized cells in each assay reflected the amount of sialic acid that was released from glycoconjugates (one unit of activity was defined as the amount of enzyme that liberated 1 nmol of sialic acid per hour at 37°C) and was measured either fluorometrically when 4-MU-NANA was used or by HPLC when gangliosides or endogenous sialylconjugates were used. In the absence of 4-MU-NANA and exogenous gangliosides, 3.9 ± 1.0 nmol of sialic acid were liberated per hour by the sialidase activity in 1 mg of total protein from freshly isolated monocytes (day 0, Fig. 1A). The amount of this activity against endogenous substrates per milligram of protein rose to 17.2 ± 3.7 units when these cells had differentiated into macrophages after 7 days in culture (day 7, Fig. 1A). The 22.2 ± 2.3 units of sialidase activity in freshly isolated monocytes detected when exogenous gangliosides were used as substrate increased to 48.1 ± 4.4 units after 7 days in culture (Fig. 1B). With 4-MU-NANA as substrate, 4.7 ± 1.2 units of sialidase activity in freshly isolated monocytes rose to 64.0 ± 9.7 units after 7 days in culture (Fig. 1C). Sialidase activity was not detected in monocytes or monocyte-derived macrophages when the assay measuring activity against endogenous sialylconjugates (i.e. in the absence of 4-MU-NANA or exogenous gangliosides) was performed at 4°C, making it unlikely that the liberated sialic acid that was measured in this condition (Fig. 1A) was simply the result of free intracellular sialic acid being released from solubilized cells (data not shown). These results using different substrates demonstrate that the endogenous sialidase activity of monocytes increases as they differentiate in vitro into macrophages.

The increase in activity of lysosomal sialidase Neu1 during monocyte differentiation is greater than the change in activity of other lysosomal enzymes

Neu1 exists in a multienzyme complex with β-d-galactosidase and cathepsin A in the lysosome and when isolated from solubilized cells (reviewed in [18,31–34]). To determine whether Neu1 was responsible for most of the activity seen with 4-MU-NANA in Fig. 1C, antibodies to human cathepsin A were used to coimmunoprecipitate Neu1 from the cell lysate prior to evaluating sialidase activity. The anti-cathepsin A Igs immunoprecipitated most of the β-galactosidase (GAL) activity from both monocytes and macrophages, whereas β-hexosaminidase (HEX) activity, that is not associated with the Neu1 multienzyme complex, was not changed (Fig. 2). These antibodies precipitated from both monocyte and macrophage extracts more than 99% of sialidase activity against 4-MU-NANA at pH 4.4 (Fig. 2). When cell extracts were incubated in the presence of preimmune Igs prior to immunoprecipitation, there was no change in the amount of sialidase activity against 4-MU-NANA (data not shown). The anti-cathepsin antibodies did not remove the sialidase activity against mixed gangliosides (MG, Fig. 2), suggesting that the sialidase activity measured with mixed bovine gangliosides was not due to the activity of Neu1. Thus, the activity of Neu1 and at least one other sialidase increased during monocyte differentiation into macrophages.

To determine whether the activity of Neu1 was specifically up-regulated during monocyte differentiation, changes in activity of other lysosomal enzymes and in the amount of a specific lysosomal protein (LAMP-2) were also measured as freshly isolated monocytes differentiated into macrophages. The specific activity of sialidase using 4-MU-NANA as substrate increased 12- to 14-fold during monocyte differentiation into macrophages (Fig. 1C and Table 1). In contrast, the specific activity of other lysosomal enzymes (β-hexosaminidase, β-galactosidase and cathepsin A) and the amount of the lysosomal membrane protein LAMP-2 increased only two to four fold during differentiation of monocytes to macrophages (Table 1). In addition, the specific activity of the mitochondrial enzyme glutamate dehydrogenase and plasma membrane alkaline phosphatase increased 3.8- and 3.2-

fold, respectively, as monocytes differentiated into macrophages. Thus, the increase in sialidase activity during monocyte differentiation exceeded the changes in specific activity and amount of increase in other lysosomal proteins. As most of the sialidase activity measured using 4-MU-NANA under the conditions stated above represented the activity of Neu1, these results suggest that the activity of Neu1 was specifically up-regulated during monocyte differentiation.

The amount of RNA encoding Neu1 and Neu3 sialidases increases during monocyte differentiation

To determine whether the increased sialidase activity in monocyte-derived macrophages that was seen using various substrates (Fig. 1A–C) was associated with increased expression of RNA encoding Neu1, Neu2, Neu3, and Neu4, the relative amount of these RNAs in freshly isolated monocytes and in macrophages maintained in culture over a 7-day period was determined by real-time RT-PCR. The amount of RNA for each sialidase was compared with the amount of RNA encoding 18S rRNA, an internal control for gene expression in the differentiating monocytes. RNAs encoding Neu1, Neu3, and Neu4 were detected in freshly isolated monocytes and monocyte-derived macrophages, but no RNA encoding Neu2 was detected in either cell (data not shown). As monocytes differentiated into macrophages, the amount of RNA encoding Neu1 and Neu3 increased 3.5 ± 0.2- and 3.9 ± 0.8-fold, respectively, in relation to the change in amount of 18S rRNA (Fig. 3). In contrast, the amount of *Neu4*-specific RNA declined 6.7 ± 0.1-fold during differentiation (Fig. 3). At all times analyzed, the absolute amount of *Neu1* RNA exceeded that of *Neu3* and *Neu4* (crossover thresholds C_T during PCR for 18S rRNA, *Neu1*, *Neu3*, and *Neu4* RNAs in monocytes were 17.7 ± 0.1, 26.1 ± 0.4, 29.5 ± 0.5, and 27.8 ± 0.6, respectively). The results were specific for each gene as confirmed by the expected size and characteristic melting temperature of each PCR gene product (data not shown).

The amount of Neu1 and Neu3 proteins increases during differentiation of monocytes to macrophages

Given the increase in sialidase activity and in amount of RNA encoding Neu1 and Neu3 that occurred when monocytes differentiated to macrophages, it was determined whether there was a corresponding increase in the total amount of Neu1 and Neu3 proteins. Proteins from freshly isolated monocytes and from monocyte-derived macrophages were separated by SDS/PAGE and then analyzed on western blots using rabbit polyclonal antibodies that were specific for Neu1 and for Neu3. The anti-Neu1 IgGs recognized the 44–46 kDa Neu1 sialidase in monocytes and macrophages (Fig. 4A). As expected from the observed increase in Neu1-specific RNA and in sialidase activity using 4-MU-NANA, immuno-detection of Neu1 with anti-Neu1 IgGs revealed a more intense band in macrophages than in monocytes (Fig. 4A). Likewise, the anti-Neu3 IgGs recognized a protein with molecular mass of 47 kDa in both monocytes and macrophages (Fig. 4B), with an increase in intensity of staining of this protein in macrophages (Fig. 4B). Thus, these results suggest that the absolute amounts of both Neu1 and Neu3 proteins increased as monocytes differentiated into macrophages, consistent with an increase in the amount of RNA encoding each.

DISSUSSION

We have described in this report that endogenous sialidase activity of freshly isolated human monocytes increases as cells differentiate in vitro into macrophages. The 12- to 14-fold increase in specific activity of sialidase in macrophages measured using 4-MUNANA reflected predominantly the activity of Neu1 sialidase. This was confirmed by the removal of greater than 99% of sialidase activity using 4-MU-NANA when Neu1 was immunoprecipitated from the cell lysate using antibodies to cathepsin A as was described previously [34]. The increase in Neu1 activity during monocyte differentiation was consistent with the observed increase in *Neu1*-specific RNA and in Neu1 protein, as shown by real time RT-PCR and western blot analyses. This increase in Neu1 activity during monocyte differentiation was at least threefold

greater than the change in specific activity of other lysosomal proteins, suggesting that the expression of Neu1 was specifically up-regulated. It remains to be determined whether the increased enzymatic activity of Neu1 in monocyte-derived cells results simply from increased transcription of *Neu1* RNA. Although there was only a 3.5-fold increase in *Neu1*-specific RNA in macrophages, there was greater than a 12-fold increase in enzymatic activity. This apparent discrepancy between amount of RNA and enzyme activity was likely not due to changes in the expression of cathepsin A, as the specific activity of cathepsin A increased only 1.8-fold in macrophages compared to monocytes. Cathepsin A, also referred to as protective protein / cathepsin A (PPCA), is a protein component of the 1.27 MDa Neu1 multienzyme complex that protects and activates Neu1 [reviewed in 18,31–34]. We previously have shown that cathepsin A is present in human placenta in at least 100-fold molar excess to the Neu1 sialidase. A portion (about 30%) of cathepsin A exists in the form of a 680 kDa complex with β-galactosidase [34–37], while a larger amount is present in 110 kDa homodimers. These homodimers are in dynamic equilibrium with the 1.27 MDa Neu1-containing complex, but the average ratio between the 1.27 MDa and 680 kDa complexes is 1–10 [34,35,38]. Similar data were reported for other tissues [39–43]. Therefore, it is likely in monocyte-derived cells that there is an excess of cathepsin A to stabilize and activate the amount of Neu1 that is present. Neu1 has the potential for post-translational modifications: it has several potential glycosylation sites and is phosphorylated in activated lymphocytes [19]. Thus, it is possible that the specific up-regulation of Neu1 activity in macrophages may result partly from post-translational modifications.

Sialidase activity was also measured using mixed bovine gangliosides under conditions that detect preferentially Neu3 sialidase [13,14,30]. The twofold increase of this activity in macrophages was consistent with the two- to fourfold increase in expression of other cellular enzymes that were analyzed. Immunoprecipitation of Neu1 from the cell lysate using anti-cathepsin A Igs had little effect on the increased sialidase activity detected with gangliosides, suggesting that this activity was not due to the activity of Neu1. The increase in sialidase activity detected with exogenous

gangliosides likely was a result of neither Neu2 nor Neu4 activity. Neu2 activity was barely detectable and the amount was unchanged in monocytes and macrophages (0.39 and 0.30 units per mg cellular protein, respectively) when measured under conditions that were specific for Neu2, and the level of *Neu4* RNA declined. The increase in the amount of *Neu3* RNAs and of the 47 kDa protein detected with anti-Neu3 IgGs support that Neu3 is responsible for this activity. The increased sialidase activity in activated cells of the immune system [2,5,6,20,24–27] has recently been attributed in lymphocytes to specific forms of sialidase [20]. Neu1 and Neu3 sialidases were found to be up-regulated in human CD4+ lymphocytes that were activated with antibodies to CD3 and CD28 [20]. As was shown previously for Neu1 [2], these sialidases appeared to play a role in cytokine production in lymphocytes [20]. Activation of the THP-1 monocytic cell line by exposure to lipopolysaccharide for at least 8–12 h also leads to enhanced sialidase activity (presumed to be Neu1), yet the specific sialidase(s) involved was not directly identified [5,27]. One effect of this enhanced activity in monocytes was increased binding of the cell surface protein CD44 to hyaluronic acid, a component of the extracellular environment [5,27]. Changes in the expression of Neu1 and Neu3 sialidases have been detected in other types of human cells that were induced to differentiate. Malignant colon cells express more *Neu3* RNA and gangliosidespecific sialidase activity than normal colonic cells, yet when these cells were induced to differentiate, the amount of *Neu3* RNA and sialidase activity declined while Neu1 activity increased [23]. It should be noted that the function of Neu3 appeared to be different in neuroblastoma cells in which the over-expression of a transfected *Neu3* gene promoted differentiation [4,21,22].

Monocytes and macrophages perform many critical functions in the immune system. During monocyte differentiation, the increase that we observed in the activity of lysosomal Neu1, especially if translocated from lysosomes to the cell surface as occurs in activated lymphocytes [19], may be important for some of these functions. Given the altered cytokine production of monocytes following desialylation of cell surface glycoconjugates [29], it is possible that the enhanced Neu1 activity may contribute to cell activation and/or differentiation. Desialylation of glycoconjugates

on the surface of monocyte-derived cells likely influences the cell to cell interactions that are critical for cell-mediated immunity. Like other cells of the immune system, monocytes and macrophages express sialic acid binding Ig-like lectins (siglecs) on their surface [reviewed in 44]. As some of these siglecs have binding sites that are masked by sialic acid on resting cells, it is possible that during monocyte differentiation, binding sites are exposed by the increased expression of Neu1. Cell-to-cell interactions that are mediated by numerous other carbohydrate recognition molecules (e.g. galectins, selectins) [reviewed in 45] could also be influenced by the action of Neu1 and Neu3 on cell surface glycoconjugates.

Macrophages recognize, phagocytize and process foreign objects (e.g. bacteria, viruses) and present antigens on the cell surface for stimulation of other cells of the immune system. Desialylation of cell surface glycoconjugates in vivo may make monocytes and macrophages more responsive to activation [29] and increase their chemotactic response to sites of inflammation, as was shown in PMNs [7]. As an antigen presenting cell, macrophages may be able to enhance the immunogenicity of processed antigens if the increased sialidase activity results in removal of the sialic acid masks of concealed epitopes [46]. In this respect, it is of interest to note that in dendritic cells, major histocompatibility class II molecules are present in the lysosome (intracellular site of Neu1) prior to translocation to the cell surface with processed antigens (reviewed in [47]).

Although we have described the expression of sialidases in monocytes and macrophages and discussed their potential role in cell function, the opposing activity of sialyltransferases, a family of enzymes that add sialic acid to the terminal galactose of glycoconjugates, can not be ignored. Hyposialylation of cell surface glycoconjugates occurs in activated cells [6,48–50], but this could occur from increased sialidase activity and/or from decreased sialyltransferase activity, as was recently demonstrated for the transmembrane protein tyrosine phosphatase CD45 [50]. Specific galactosebinding lectins have been used to characterize the sialylation status of the cell surface [6,49,50], but it should be noted that these lectins bind to glycomoieties that may represent only a fraction of total potential sialylation sites,

and thus, their binding may not reflect the global sialylation state of the cell. Further studies will define whether there is a global hyposialylation of the cell surface during monocyte differentiation or whether specific molecules are the target of the Neu1 and Neu3 sialidases.

Although the plasma-membrane and lysosomal sialidases localize predominantly to distinct intracellular sites, translocation throughout the cell occurs [7,19,26]. The lysosomal sialidase is translocated in activated lymphocytes from intracellular organelles to the cell surface after being phosphorylated by a cellular kinase [19]. It is possible that lysosomal Neu1 also is translocated to the periphery of monocyte-derived cells and, with the continuous endocytosis that occurs in these cells, that the membrane-associated Neu3 sialidase of macrophages is also recycled through the cell between the cell surface and intracellular granules. Given the changes in expression and dynamic intracellular repositioning of Neu1 and Neu3 that likely occur during monocyte differentiation, establishing the role(s) of human sialidases during the differentiation of monocytes presents great challenges.

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REFERENCES

1 Boog CJ, Neefjes JJ, Boes J, Ploegh HL & Melief CJ (1989) Specific immune responses restored by alteration in carbohydrate chains of surface molecules on antigenpresenting cells. *Eur J Immunol* 19, 537–542.

2 Chen XP, Enioutina EY & Daynes RA (1997) The control of IL-4 gene expression in activated murine T lymphocytes: a novel role for neu-1 sialidase. *J Immunol* 158, 3070–3080.

3 Stamatos NM, Gomatos PJ, Cox J, Fowler A, Dow N, Wohlhieter JA & Cross AS (1997) Desialylation of peripheral blood mononuclear cells promotes growth of HIV-1. *Virology* 228, 123–131.

4 Wu G & Ledeen RW (1991) Stimulation of neurite outgrowth in neuroblastoma cells by neuraminidase: putative role of GM1 ganglioside in differentiation. *J Neurochem* 56, 95–104.

5 Katoh S, Miyagi T, Taniguchi H, Matsubara Y, Kadota J, Tominaga A, Kincade PW, Matsukura & Kohno S (1999) Cutting edge: an inducible sialidase regulates the hyaluronic acid binding ability of CD44-bearing human monocytes. *J Immunol* 162, 5058–5061.

6 Galvan M, Murali-Krishna K, Ming LL, Baum L & Ahmed R (1998) Alterations in cell surface carbohydrates on T cells from virally infected mice can distinguish effector/memory CD8+ T cells from naı¨ ve cells. *J Immunol* 161, 641–648.

7 Cross AS, Sakarya S, Rifat S, Held TK, Drysdale BE, Grange PA, Cassels FJ, Wang LX, Stamatos NM, Farese A, Casey D, Powell J, Bhattacharjee AK, Kleinberg M & Goldblum SE (2003) Recruitment of murine neutrophils in vivo through endogenous sialidase activity. *J Biol Chem* 278, 4112–4120.

8 Hunig T (1983) The role of accessory cells in polyclonal T cell activation II. Induction of interleukin 2 responsiveness requires cell-cell contact. *Eur J Immunol* 13, 596–601.

9 Pshezhetsky AV, Richard C, Michaud L, Igdoura S, Wang S, Elsliger MA, Qu J, Leclerc D, Gravel R, Dallaire L & Potier M (1997) Cloning, expression and

chromosomal mapping of human lysosomal sialidase and characterization of mutations in sialidosis. *Nat Genet* 15, 316–320.

10 Milner CM, Smith SV, Carrillo MB, Taylor GL, Hollinshead M & Campbell RD (1997) Identification of a sialidase encoded in the human major histocompatibility complex. *J Biol Chem* 272, 4549–4558.

11 Miyagi T, Konno K, Emori Y, Kawasaki H, Suzuki K, Yasui A & Tsuik S (1993) Molecular cloning and expression of cDNA encoding rat skeletal muscle cytosolic sialidase. *J Biol Chem* 268, 26435–26440.

12 Tringali C, Papini N, Fusi P, Croci G, Borsani G, Preti A, Tortora P, Tettamanti G, Venerando B & Monti E (2004) Properties of recombinant human cytosolic sialidase HsNEU2: the enzyme hydrolyzes monomerically dispersed G_{M1} ganglioside molecules. *J Biol Chem* 279,

13 Monti E, Bassi MT, Papini N, Riboni M, Manzoni M, Venerando B, Croci G, Preti A, Ballabio A, Tettamanti G & Borsani. G (2000) Identification and expression of NEU3, a novel human sialidase associated to the plasma membrane. *Biochem J* 349, 343–351.

14 Miyagi T, Wada T, Iwamatsu A, Hata K, Yoshikawa Y, Tokuyama S & Sawada M (1999) Molecular cloning and characterization of a plasma membrane-associated sialidase specific for gangliosides. *J Biol Chem* 274, 5004–5011.

15 Comelli EM, Amado M, Lustig SR & Paulson JC (2003) Identification and expression of Neu4, a novel murine sialidase. *Gene* 321, 155–161.

16 Monti E, Bassi MT, Bresciani R, Civini S, Croci GL, Papini N, Riboni M, Zanchetti G, Ballabio A, Preti A, Tettamanti G, Venerando B & Borsani G (2004) Molecular cloning and characterization of NEU4, the fourth member of the human sialidase gene family. *Genomics* 83, 445–453.

17 Seyrantepe V, Landry K, Trudel S, Hassan JA, Morales CR & Pshezhetsky AV (2004) Neu4, a novel human lysosomal lumen sialidase confers normal phenotype to sialidosis and galactosialidosis cells. *J Biol Chem* 279, 37021–37029.

18 Pshezhetsky AV & Ashmarina M (2001) Lysosomal multienzyme complex: biochemistry, genetics, and molecular pathophysiology. *Prog Nucleic Acid Res Mol Biol* 69, 81–114.

19 Lukong KE, Seyrantepe V, Landry K, Trudel S, Ahmad A, Gahl WA, Lefrancois S, Morales CR & Pshezhetsky AV (2001) Intracellular distribution of lysosomal sialidase is controlled by the internalization signal in its cytoplasmic tail. *J Biol Chem* 276, 46172–46181.

20 Wang P, Zhang J, Bian H, Wu P, Kuvelkar R, Kung TT, Crawley Y, Egan RW & Billah MM (2004) Induction of lysosomal and plasma membrane-bound sialidases in human T-cells via T-cell receptor. *Biochem J* 380, 425–433.

21 Hasegawa T, Yamaguchi K, Wada T, Takeda A, Itoyama Y & Miyagi T (2000) Molecular cloning of mouse ganglioside sialidase and its increased expression in Neuro2a cell differentiation. *J Biol Chem* 275, 8007– 8015.

22 Kopitz J, Muhl C, Ehemann V, Lehmann C & Cantz M (1997) Effects of cell surface ganglioside sialidase inhibition on growth control and differentiation of human neuroblastoma cells. *Eur J Cell Biol* 73, 1–9.

23 Kakugawa Y, Wada T, Yamaguchi K, Yamanami H, Ouchi K, Sato I & Miyagi T (2002) Up-regulation of plasma membrane-associated ganglioside sialidase (Neu3) in human colon cancer and its involvement in apoptosis suppression. *Proc Natl Acad Sci USA* 99, 10718–10723.

24 Taira S & Nariuchi H (1988) Possible role of neuraminidase in activated T cells in the recognition of allogeneic Ia. *J Immunol* 141, 440–446.

25 Landolfi NF, Leone J, Womack JE & Cook RG (1985) Activation of T lymphocytes results in an increase in H-2-encoded neuraminidase. *Immunogenetics* 22, 159– 167.

26 Cross AS & Wright DG (1991) 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* 88, 2067–2076.

27 Gee K, Kozlowski M & Kumar A (2003) Tumor necrosis factor-alpha induces functionally active hyaluronanadhesive CD44 by activating sialidase through p38

mitogen-activated protein kinase in lipopolysaccharidestimulated human monocytic cells. *J Biol Chem* 278, 37275–37287.

28 Sallusto F & Lanzavecchia A (1994) Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte / macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J Exp Med* 179, 1109– 1118.

29 Stamatos NM, Curreli S, Zella D & Cross AS (2004) Desialylation of glycoconjugates on the surface of monocytes activates the extracellular signal-related kinases ERK 1 / 2 and results in enhanced production of specific cytokines. *J Leuk Biol* 75, 307–313.

30 Wang Y, Yamaguchi K, Shimada Y, Zhao X & Miyagi T (2001) Site-directed mutagenesis of human membraneassociated ganglioside sialidase: identification of aminoacid residues contributing to substrate specificity. *Eur J Biochem* 268, 2201–2208.

31 van der Horst GT, Galjart NJ, d'Azzo A, Galjaard H & Verheijen FW (1989) Identification and in vitro reconstitution of lysosomal neuraminidase from human placenta. *J Biol Chem* 264, 1317–1322.

32 Tranchemontagne J, Michaud L & Potier M (1990) Deficient lysosomal carboxypeptidase activity in galactosialidosis. *Biochem Biophys Res Commun* 168, 22-29.

33 Galjart NJ, Morreau H, Willemsen R, Gillemans N, Bonten NJ & d'Azzo A (1991) Human lysosomal protective protein has cathepsin A-like activity distinct from its protective function. *J Biol Chem* 266, 14754–14762.

34 Pshezhetsky AV & Potier M (1994) Direct affinity purification and supramolecular organization of human lysosomal cathepsin A. *Arch Biochem Biophys* 313, 64–70.

35 Pshezhetsky AV & Potier M (1996) Association of N-acetyl-galactosamine-6-sulfate sulfatase with the multienzyme lysosomal complex of b-galactosidase, cathepsin A and neuraminidase: possible implication for intralysosomal catabolism of keratan sulfate. *J Biol Chem* 271, 28359–28365.

36 Pshezhetsky AV & Potier M (1993) Stoichiometry of the human lysosomal carboxypeptidase-b-galactosidase complex. *Biochem Biophys Res Commun* 195, 354–362.

37 Pshezhetsky AV, Elsliger M-A, Vinogradova MV & Potier M (1995) Human lysosomal b-galactosidase-cathepsin A complex: definition of the β-galactosidase-binding interface on cathepsin A. *Biochem* 34, 2431–2440.

38 Vinogradova MV, Michaud L, Mezentsev AV, Lukong KE, El-Alfy M, Morales CR, Potier M & Pshezhetsky AV (1998) Molecular mechanism of lysosomal sialidase deficiency in galactosialidosis involves its rapid degradation. *Biochem J* 330, 641–650.

39 Verheijen F, Palmeri S, Hoogeveen AT & Galjaard H (1985) Human placental neuraminidase: activation, stabilization and association with b-galactosidase and its 'protective' protein. *Eur J Biochem* 149, 315–321.

40 Hiraiwa M, Uda Y, Nishizawa M & Miyatake T (1987) Human placental sialidase: partial purification and characterization. *J Biochem* (Tokyo) 101, 1273–1279.

41 Hiraiwa M, Nishizawa M, Uda Y, Nakajima T & Miyatake. T (1988) Human placental sialidase: further purification and characterization. *J Biochem* (Tokyo) 103, 86–90.

42 Verheijen FW, Palmeri S & Galjaard H (1987) Purification and partial characterization of lysosomal neuraminidase from human placenta. *Eur J Biochem* 162, 63–67.

43 Hiraiwa M, Saitoh M, Arai N, Shiraishi T, Odani S, Uda Y, Ono T & O'Brien JS (1997) Protective protein in the bovine lysosomal β-galactosidase complex. *Biochim Biophys Acta* 1341, 189–199.

44 Crocker PR & Varki A (2001) Siglecs in the immune system. *Immunology* 103, 137–145.

45 Sharon N & Lis H (2004) History of lectins: from hemagglutinins to biological recognition molecules. *Glycobiology* 14, 53R–62R.

46 Benjouad A, Gluckman JC, Montagnier L & Bahraoui E (1993) Specificity of antibodies produced against native or desialylated human immunodeficiency virus type 1 recombinant gp160. *J Virol 67*, 1693–1697.

47 Mellman I & Steinman RM (2001) Dendritic cells: specialized and regulated antigen processing machines. *Cell* 106, 255–258.

48 Landolfi NF & Cook RG (1986) Activated T-lymphocytes express class I molecules which are hyposialylated compared to other lymphocyte populations. *Mol Immunol* 3, 297–309.

49 Lefebvre JC, Giordanengo V, Doglio A, Cagnon L, Breittmayer JP, Peyron JF & Lesimple J (1994) Altered sialylation of CD45 in HIV-1-infected T lymphocytes. *Virology* 199, 265–274.

50 Amado M, Yan Q, Comelli EM, Collins BE & Paulson JC (2004) Peanut agglutinin high phenotype of activated CD8+ T cells results from de novo synthesis of CD45 glycans. *J Biol Chem* 279, 36689–36697.

51 Rome LH, Garvin AJ, Allietta MM & Neufeld EF (1979) Two species of lysosomal organelles in cultured human fibroblasts. *Cell* 17, 143–153.

52 Okada S & O'Brien JS (1968) Generalized gangliosidosis: beta-galactosidase deficiency. *Science* 160, 1002– 1004.

53 Bergmeyer H, Gawehn K & Grassl M (1974) Alkaline Phosphatase. *In Methods of Enzymatic Analysis* (Bergmeyer HV, ed), Vol. 1 2nd English, pp. 495–496. Verlag-Chemie, Weinheim, Federal Republic of Germany.

54 Schmidt E (1974) Glutamate dehydrogenase. *In Methods of Enzymatic Analysis* (Bergmeyer HV, ed), Vol. 1 2nd English, pp. 650–656. Verlag-Chemie, Weinheim, Federal Republic of Germany.

55 Miyagi T, Sagawa J, Konno K & Tsuiki S (1990) Biochemical and immunological studies on two distinct ganglioside-hydrolyzing sialidases from the particulate fraction of rat brain. *J Biochem* 107, 787–793.

56 Miyagi T & Tsuiki S (1985) Purification and characterization of cytosolic sialidase from rat liver. *J Biol Chem* 260, 6710–6716.

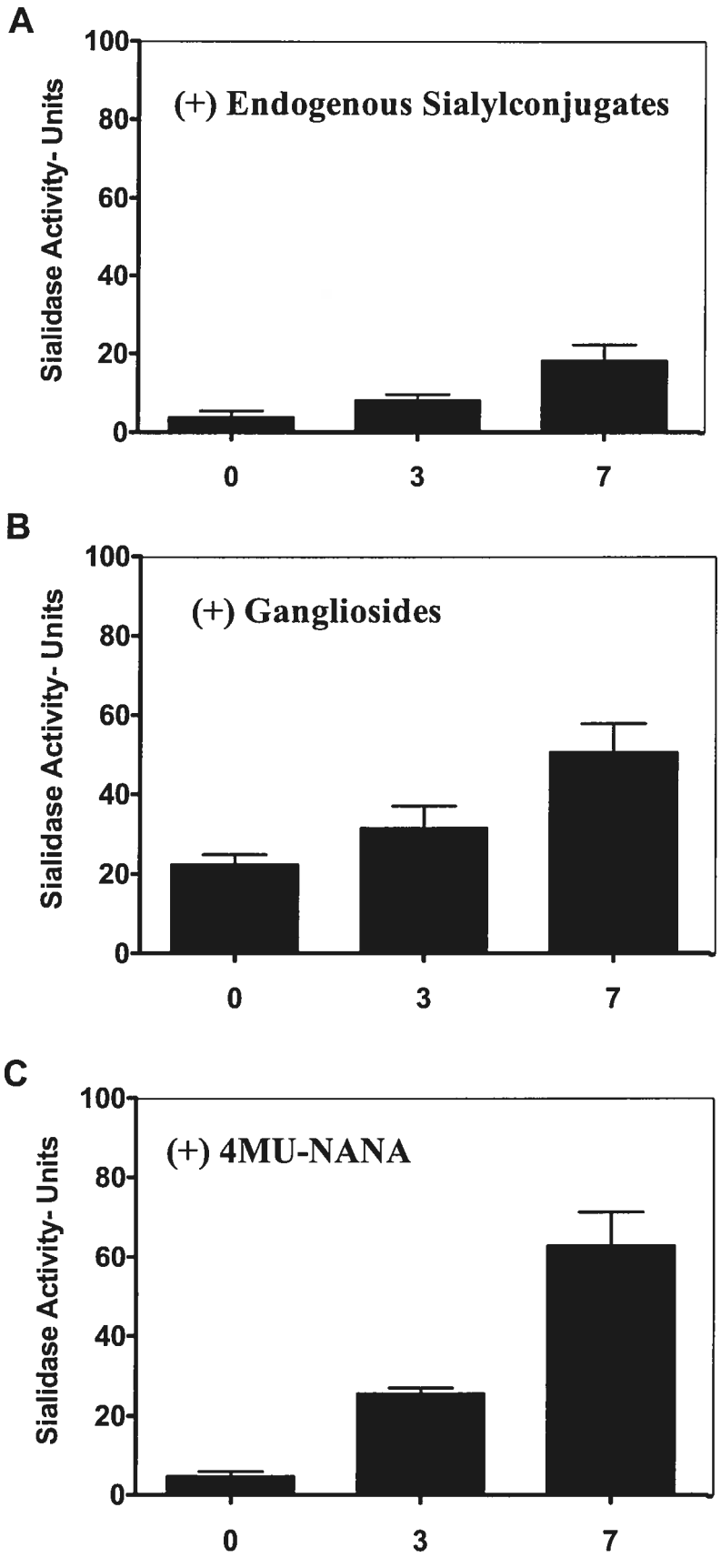
A
(+) Endogenous Sialylconjugates
Sialidase Activity- Units
100
80
60
40
20
0
0
3
7
B
(+) Gangliosides
Sialidase Activity- Units
100
80
60
40
20
0
0
3
7
C
(+) 4MU-NANA
Sialidase Activity- Units
100
80
60
40
20
0
0
3
7

Figure 1

Figure 1 Differentiation of monocytes into macrophages is associated with increased expression of endogenous sialidase.

Monocytes were purified from the peripheral blood of human donors as described in Experimental procedures and were differentiated into macrophages by growth at 37°C in RPMI medium 1640 with 10% (v/v) human serum and rhM-CSF. Sialidase activity in cells from three donors was determined immediately after isolation of monocytes (day 0) and after cells had differentiated in culture for 3 and 7 days. Sialidase activity was measured against endogenous sialylconjugates (A), mixed bovine gangliosides (B), or 4-MU-NANA (C) as substrates as described in Experimental procedures. Sialidase activity is reported in units that reflect the amount of sialidase in 1 mg of cellular protein that releases 1 nmol of sialic acid per hour at 37°C. Data represent the mean ± SE of three independent experiments using cells from three different donors.

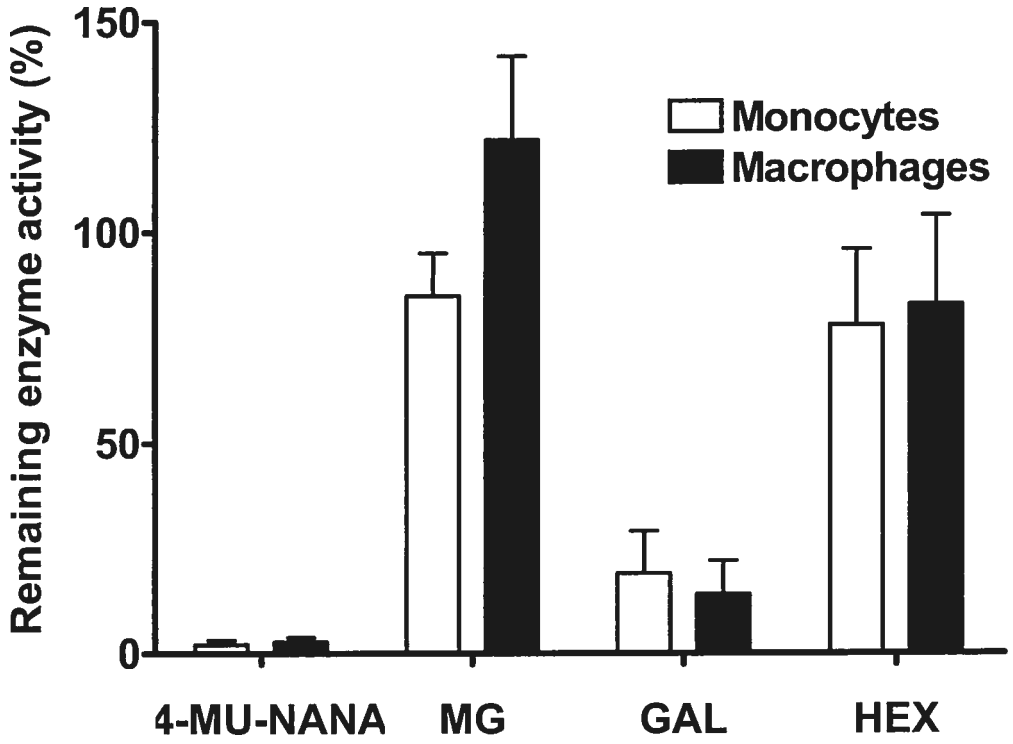
Remaining enzyme activity (%)
150
100
50
0
Monocytes
Macrophages
4-MU-NANA
MG
GAL
HEX

Figure 2

Figure 2. Immunoprecipitation of Neu1 from cell extracts removes sialidase activity using 4-MU-NANA as substrate.

Monocytes and monocyte-derived macrophages were isolated, homogenized and incubated with rabbit anti-cathepsin A IgG or preimmune IgG as described in Experimental procedures. After immunoprecipitation of the Neu1-containing multienzyme complex that also contains β-D-galactosidase and cathepsin A, the depleted lysate was assayed for β-galactosidase (GAL), β-hexosaminidase (HEX), and sialidase activities using either 4-MU-NANA or mixed gangliosides (MG) as substrates as described in Experimental procedures. The amount of activity of each enzyme in the presence of preimmune IgG was set to 100% of activity for comparison with the activity in the samples treated with anti-cathepsin A IgG. Data represent the mean ± SE of three independent experiments.

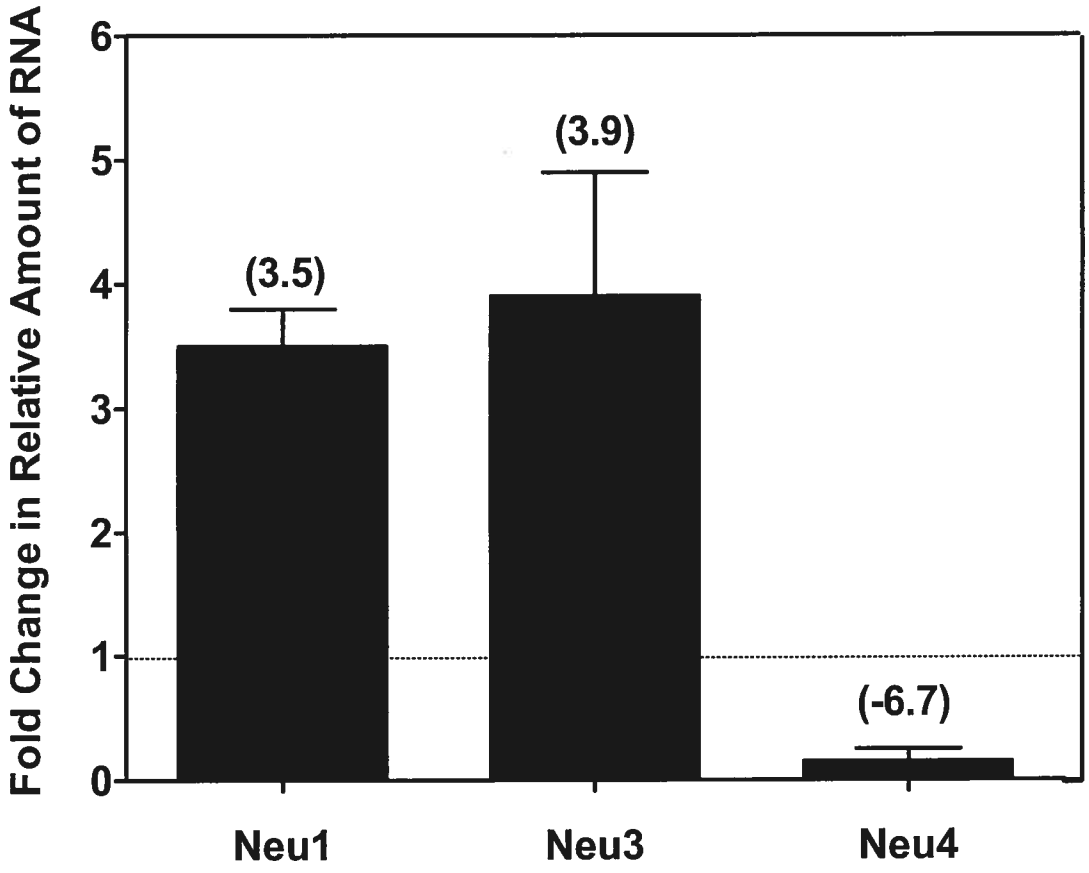
Fold Change in Relative Amount of RNA
6
5
4
3
2
1
0
(3.5)
(3.9)
(-6.7)
Neu1
Neu3
Neu4

Figure 3

Figure 3. Differential regulation of genes encoding Neu1, Neu3 and Neu4 during monocyte differentiation.

Total RNA was isolated from monocytes and monocyte-derived macrophages after 7 days in culture and 10 ng of RNA was used with primers that were specific for Neu1–4 in SYBR-green semi-quantitative real-time RT-PCR to detect the relative amount of RNA encoding each gene as described in Experimental procedures. The fold change in amount of *Neu1*, *Neu3* and *Neu4* RNAs in day 7 macrophages compared to freshly isolated monocytes (listed in parentheses) was calculated after normalization to the internal control 18S rRNA by the equation $2^{-\Delta\Delta CT}$ as described in Experimental procedures. The difference in amount of expression of each gene relative to 18S rRNA in monocytes was normalized to 1, as noted by the dotted horizontal line at 1. These data represent the mean ± SE of three experiments using cells from different donors.

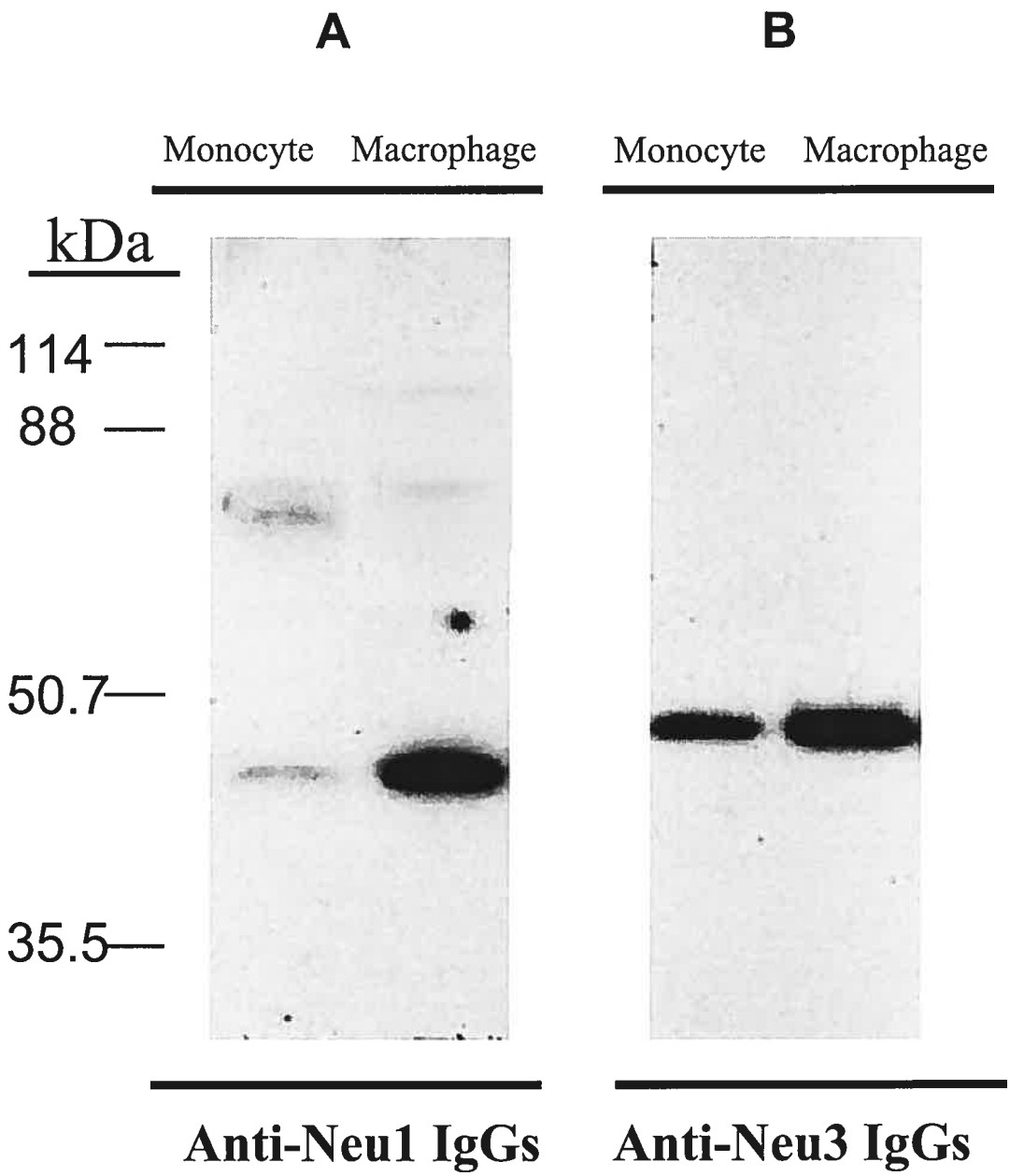
A
B
Monocyte
Macrophage
Monocyte
Macrophage
kDa
114
88
50.7
35.5
Anti-Neu1 IgGs
Anti-Neu3 IgGs

Figure 4

Figure 4. The amount of Neu1 and Neu3 proteins increases during monocyte differentiation.

Monocytes and macrophages were collected at the indicated times and total cellular protein was separated by electrophoresis on 10% SDS/polyacrylamide gels, transferred to polyvinyldifluoride membranes and analyzed for the total amount of Neu1 (A) and Neu3 (B) protein using specific antibodies as described in Experimental procedures. The same amount of total cellular protein (5 μg) from both monocytes and macrophages was analyzed in each lane of the gel. The tick marks on the left side of the radiograph represent protein molecular mass markers as noted. These results from one donor are representative of data from five independent experiments using cells from four different donors.

Table 1. Specific activity and amount of select proteins in monocytes and macrophages.

Proteins	Specific activity and amount	
	Monocytes	Macrophages
Sialidase	3.5 ± 1.4	42.5 ± 8.9 (*12.1*)
β-Hexosaminidase	1434 ± 96	4476 ± 595 (*3.1*)
β-Galactosidase	368 ± 10	1352 ± 16 (*3.7*)
Cathespin A	3210 ± 154	5720 ± 617 (*1.8*)
LAMP-2	100.0 ± 8.5 (relative units)	380.1 ± 21 (*3.8*) (relative units)
Glutamate dehydrogenase	127.4 ± 33.9	482.5 ± 20.2 (*3.8*)
Alkaline phosphatase	1.93 ± 0.64	6.08 ± 0.69 (*3.2*)

CHAPTER 3: MONOCYTE DIFFERENTIATION UPREGULATES THE EXPRESSION OF THE LYSOSOMAL SIALIDASE, NEU1 AND TRIGGERS ITS TARGETING TO THE PLASMA MEMBRANE VIA MHC CLASS II-POSITIVE COMPARTMENTS.

ARTICLE 2

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MONOCYTE DIFFERENTIATION UPREGULATES THE EXPRESSION OF THE LYSOSOMAL SIALIDASE, NEU1 AND TRIGGERS ITS TARGETING TO THE PLASMA MEMBRANE VIA MHC CLASS II-POSITIVE COMPARTMENTS.

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ABSTRACT

Human sialidase (neuraminidase) Neu1 catalyzes lysosomal catabolism of sialylated glycoconjugates. Here we show that during the differentiation of monocytes and the monocytic cell line, THP-1 into macrophages, the majority of Neu1 relocalizes from the lysosomes to the cell surface. In contrast to other cellular sialidases Neu2, Neu3 and Neu4, whose expression either remains unchanged or is downregulated, *Neu1* mRNA, protein and activity are specifically increased during the PMA-induced differentiation, consistent with a significant induction of the transcriptional activity of the *Neu1* gene promoter. The lysosomal carboxypeptidase, cathepsin A, which forms a complex with and activates Neu1 in the lysosome, is sorted to the plasma membrane of the differentiating cells similarly to Neu1. Both proteins are first targeted to the lysosome and then are sorted to the LAMP-2-negative, MHC II positive vesicles, which later merge with the plasma membrane. Similar trafficking was observed for the internalized fluorescent dextran or horseradish peroxidase initially stored in the lysosomal/endosomal compartment. The suppression of Neu1 expression in the THP-1-derived macrophages by siRNA or with anti-Neu1 antibodies significantly reduced the ability of the cells to engulf bacteria or to produce cytokines. Altogether our data suggest that the upregulation of the Neu1 expression is important for the primary function of macrophages and establish the link between Neu1 and the cellular immune response.

INTRODUCTION

Human lysosomal sialidase (neuraminidase) Neu1, encoded by the *NEU1* gene in the MHC III locus (1-3) catalyzes the hydrolytic cleavage of terminal sialic acid residues from oligosaccharides and glycoproteins. In the lysosome, Neu1 exists as a component of the multienzyme complex also containing the lysosomal carboxypeptidase A (cathepsin A/protective protein), ß-galactosidase and Nacetylgalactosamine- 6-sulfate sulfatase [reviewed in (4,5)]. The dissociation of the complex *in vitro* results in the reversible inactivation of Neu1 (6). *In vivo*, inherited mutations in cathepsin A cause disruption of the complex and trigger galactosialidosis (OMIM 256540), an autosomal recessive disease characterized by combined deficiency of Neu1, ß-galactosidase and cathepsin A [reviewed in (4)]. Another autosomal recessive disease, sialidosis (OMIM 256550), is caused by mutations directly affecting the *NEU1* gene [reviewed in (7,8)].

We have previously shown that in addition to the lysosomes, Neu1 is also present on the surface of activated T cells (9) where it may influence the immune function (10-12). Recently (13) we have demonstrated that endogenous Neu1 sialidase activity of freshly isolated human monocytes is increased 20 to 30-fold per cell as they differentiate into macrophages. Monocyte-macrophages constitute an important cellular component of the mononuclear phagocytic system, playing an essential role in the recognition and activation of both innate and adaptive immune responses against pathogens. The differentiation of monocytes into macrophages in extravascular tissues is an essential event for the normal functioning of the system. Therefore, knowledge of the mechanism and the role of the *Neu1* upregulation during the monocyte differentiation should provide insights into the cellular immune response and may eventually lead to developing approaches for therapeutic intervention.

Here we have studied the regulatory mechanisms of the Neu1 expression and presented direct evidence that the *NEU1* gene promoter is activated during the monocytic differentiation. We have also shown that the newly synthesized Neu1 is first targeted to the lysosomes and then, as a complex with cathepsin A, relocalizes to

the cell surface, where it could participate in antigen presentation or affect intercellular interactions.

EXPERIMENTAL PROCEDURES

Isolation of PBMCs and purification of monocytes

Peripheral blood mononuclear cells (PBMCs) were isolated by leukophoresis of blood from HIV-1 and hepatitis B and C seronegative donors followed by centrifugation over Ficoll- Paque Plus (Amersham Biosciences, Uppsala, Sweden) gradients using standard procedures. Monocytes were purified from PBMCs by an additional centrifugation over Percoll (Amersham Biosciences, Uppsala, Sweden) gradients and then by negative selection using monocyte separation kit (StemSep; Stem Cell Technologies, Vancouver, BC, Canada) according to the manufacturer's protocol. The purity of monocytes exceeded 95% as determined by flow cytometry after staining the cells with fluorochrome-labeled antibodies to CD3, CD14, CD19, CD206 (all from BD PharMingen, San Diego, CA) and viability was greater than 97% as determined by trypan blue dye exclusion.

Culturing of purified monocytes

To obtain monocyte-derived macrophages, purified monocytes were suspended at 2 x 10^6 cells/ml in RPMI medium 1640 (Gibco, Grand Island, NY) containing 10% heat-inactivated human AB serum (Gemini Bioproducts, Calabasas, CA) and were maintained at 4 x 10^6 cells/well in 6 well tissue culture plates (Costar, Corning Inc., Corning, NY) at 37°C in a 5% humidified, CO_2 incubator. At the indicated times, nonadherent cells were removed by two washes with phosphate-buffered saline (PBS) and the adherent, differentiating macrophages (larger and more granular than monocytes as seen by light microscopy) were harvested in PBS pH 7.4 by gentle scraping with a polyethylene cell scraper (Nalge Nunc International, Rochester NY). The harvested cells were confirmed to have characteristic macrophage cell surface phenotypic markers (CD14+, CD206+) by flow cytometry.

Culturing and differentiation of THP-1 cells

THP-1 cells, described in greater detail elsewhere (14), were cultured in the RPMI 1640 medium (Gibco, Grand Island, NY) containing 10% heat-inactivated fetal calf serum (FCS), 5000 units of Penicillin/Streptomycin and Fungizone (both Life Technologies, Inc.). The cells were maintained in suspension at 0.5-1.0 x 10^6 cells per ml. The differentiation of THP-1 cells into adherent cells with macrophage-like characteristics widely used as a convenient *in vitro* model for macrophage differentiation (15, 16) was induced by the addition of PMA (10-20 ng per ml) to the culture medium. After 4 days the adherent, differentiating macrophages were harvested by scraping as above.

Promoter activity assay

The fragments of the 5`-flanking region of the *NEU1* gene were obtained by the digestion of pBluescript-SP vector with *Bgl*II (position -1), *Bcl*I (position -176), *Sac*II (position –316), *Xma*I (position –542), *Xba*I (position -936) and *Apa*I (position –1598). The fragments were subcloned into pBLCAT6 vector (15), provided by Dr. L. Simard, (Montreal University) in front of a chloramphenicol acetyl transferase (CAT) gene resulting in pBL-NEU1CAT1, pBL-NEU1CAT2, pBL-NEU1CAT3, pBL-NEU1CAT4 and pBLNEU1CAT5 constructs. pCMV–β–gal vector containing β–galactosidase reporter gene has been provided by Dr. R. Gravel (University of Calgary).

Monkey kidney COS-7 cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 in Modified Eagle`s medium (MEM, Life Technologies, Inc.) supplemented with 10% FCS, 5000 units of Penicillin/Streptomycin and Fungizone. Cells seeded in 100 mm tissue culture plates at 70-80% confluence were cotransfected with the reporter constructs and pCMV–β–gal expression vector using Lipofectamine Plus reagent (Life Technologies Inc, Gaithersburg, MD) in accordance with manufacturer's protocol. THP-1 cells suspended in RPMI 1640 medium at a density of 5x 10^5 cells/ml medium were cotransfected with the reporter constructs

and pCMV−β−gal expression vector using Effectene transfection kit (Qiagen, Germay) in accordance with manufacturer's protocol.

Twenty-four hours after the transfection the cells were washed with PBS and stimulated for 2-24 h with PMA (20 ng/ml), TNF-α (40 ng per ml) or PDGF-AB (10 ng per ml) in the presence or absence of 20 mM NAC, or 0.5 μM curcumin (all Sigma, Ste-Louise, MO). In a separate experiment curcumin and NAC were added in the same final concentration 1 h before the addition of PMA or TNF-α. After 24 h incubation the cells were rinsed with PBS, harvested by scraping and resuspended in 350 μl of TEN buffer (50 mM Tris-HCl pH 7.4, 1 mM EDTA, and 150 mM NaCl). Cells were centrifuged at 12,000 rpm at 4°C for 5 min and lysed by three freezing-thawing cycles in 0.25 M Tris-HCl pH 7.8. CAT and β−galactosidase activities were measured using [^{14}C]-choramphenicol and 4-methylumbelliferyl− β−D-galactopyranoside substrates as described (16). One unit of enzyme activity (U) is defined as the conversion of 1 μmol of substrate/min. Proteins were assayed according to Bradford (17) with bovine serum albumin (Sigma) as standard.

Enzyme assays

Sialidase, β−galactosidase and β−hexosaminidase activities in cellular homogenates and in culture medium were assayed using the corresponding fluorogenic 4-methylumbelliferyl-glycoside substrates (18,19). Cathepsin A activity was determined with CBZPhe-Leu (20). Alkaline phosphatase, glutamate dehydrogenase and GAPDH were measured as described elsewhere (21-23). Sialidase activity towards mixed bovine gangliosides was measured as described (24,25) in the presence of 0.2% Triton X-100. The concentration of released sialic acid was measured by thiobarbituric method (26). Enzyme activity is expressed as the conversion of 1 nmol of substrate per h.

To measure the enzyme activities on the outer cell surface, the cultured live cells (~10^6 cells) were washed several times with Hank's balanced salt solution and overlaid with 500 μl of 20 mM acetate buffer, pH 5.2, 0.25 M sucrose and either 0.4 mM sialidase substrate, cathepsin A substrate, β-galactosidase substrate or N-acetyl

β-hexosaminidase substrate. After 30 min of incubation, 200 µl aliquots of medium were collected and the concentrations of the products were measured as described above. The integrity of plasma membrane was verified by western blot by measuring the amount of the cytosolic enzyme GAPDH released into the cell medium during the incubation. We found that the same amount of GAPDH was released from the cells incubated in 20 mM acetate buffer, pH 5.2, 0.25 M sucrose and PBS. To measure the total amount of enzyme, the assay was performed in the presence of 0.3% Triton X-100.

Detection of CathA and Neu1 on the macrophage surface by Western blot

Proliferating THP-1 cells and THP-1-derived macrophages (~$5x10^6$ cells) were washed 3 times by ice-cold PBS and incubated at room temperature for 1 h in 5 ml of PBS containing a BAC-SulfoNHS biotinylation reagent (Sigma St. Louis, MO) at a final concentration of 5mg/ml with a gentle rotation. The cells were washed 3 times with TBS and homogenized in 0.5 ml RIPA buffer. The cell lysate cleared by 10 min centrifugation at 12,000 g was loaded on a 0.2 ml Streptavidin-agarose column (Sigma St. Louis, MO) and the column was washed by PBS until the absorbance at 280 nm reached the baseline. Biotinylated proteins were eluted with 0.2 ml of 0.1 M glycine-HCl (pH 2.5). The pH of the eluate was immediately adjusted to neutral with 1.5 M Tris buffer (pH 7.5). Twenty µg of protein from the original cell lysate and the Streptavidin-agarose column flow-through fraction and 5 µg of protein from the eluate were separated on a NuPAGE 4-12% Bis-Tris gel (Invitrogen) and transfered to a PVDF membrane. The membrane was hybridized with the polyclonal anti-Neu1 antibodies (dilution 1:5,000) and anti-cathepsin A antibodies (dilution 1:2,000). Detection was performed using a Roche Western kit.

Internalization of Texas Red-labeled Dextran and HRP

THP-1 cells suspended at 1.0x 10^6 cells / ml in RPMI 1640 containing 10% FCS in 6-well tissue culture plate were loaded with Texas red-labeled flurescent dextran 40,000 (Molecular probe, Oregon US) in a concentration of 50 µg/ml

medium or the horseradish peroxidase (Sigma) in a concentration of 2 mg/ml medium. Treated THP-1 cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 for 2 hours. Then the cells were harvested by a low-speed centrifugation and washed with RPMI 1640 medium two times at room temperature and resuspended in the fresh medium containing 10% FCS. After 16 h of incubation at 37°C cells were treated with PMA (20 ng/ml of culture medium) as described above.

Confocal immunofluorescence microscopy

Cells were washed twice with ice-cold PBS and fixed with 3.8% paraformaldehyde in PBS for 30 min and if necessary were permeabilized by 0.5% Triton X-100. Fixed cells were washed twice with PBS and stained with polyclonal rabbit anti-Neu1, or anti-cathepsin A antibodies and counterstained with Texas Red labeled antibodies against rabbit IgG. Cells were also double-stained with rabbit antibodies against Neu1 or cathepsin A antibodies and monoclonal antibodies against LAMP-2 (Washington Biotechnology Inc., Baltimore), or against MHC II (BD Biosciences, Mississauga, ON). The primary monoclonal antibodies were counterstained with Oregon green 488-conjugated anti-mouse IgG antibodies (Molecular Probes, Eugene, OR). The cells loaded with the horseradish peroxidase (HRP) were stained with the Texas Red-conjugated antibodies against HRP (Jackson ImmunoResearch, West Grove, PA). Cells were also stained with the rabbit anti-Neu1 or anti-cathepsin A antibodies counterstained with Oregon green 488-conjugated anti-rabbit IgG antibodies and PE-conjugated monoclonal anti-mannose receptor antibodies or anti-CD14 antibodies (BD Pharmingen, USA). Slides were examined on a Zeiss LSM510 inverted confocal microscope (Carl Zeiss Inc., Thornwood).

Western blot analysis

Proteins were resolved by SDS-polyacrylamide gel electrophoresis and electrotransferred to PVDF membrane. Neu1 and Neu3 detection was performed with rabbit antibodies as described (13,27) and LAMP-2 detection with monoclonal anti-

human LAMP-2 antibodies (Washington Biotechnology Inc., Baltimore) using the BM Chemiluminiscence kit (Roche Diagnostics, Laval, QC) in accordance with the manufacturer's protocol.

RNA isolation and real time RT-PCR

THP-1 cells and THP-1-derived macrophages were harvested as previously described and total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) following the protocol suggested by the manufacturer. The RNA preparation was treated with DNase I (Invitrogen, Carlsbad, CA) for 15 minutes at room temperature to remove the DNA contamination and then inactivated by adding 25 mM EDTA solution to the reaction mixture. One microgram of RNA was used to synthesize cDNA in the presence of 0.5 mM dNTPs, 50 ng random hexamers (Invitrogen, Carlsbad, CA) and 200 Units of SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA.). Reverse transcription was allowed to proceed for 50 minutes at 42°C. PCR amplification was performed on a SmartCycler (Cepheid) using the SYBR Green PCR kit (Qiagen). The primer pairs, annealing temperatures, number of PCR cycles, and RNA amounts are shown in Supplemental Table, which also shows the predicted sizes of the resulting PCR products. As positive controls for real-time PCR detection, we used mRNA coding for the ribosomal subunit 18S (Ambion). All reactions were performed in triplicate. The accuracy of the reaction was monitored by the analysis of melting curves and product size on gel electrophoresis.

Northern blot analysis

Purified total RNA from THP-1 cells and THP-1-derived macrophages was resolved by agarose gel electrophoresis and blotted to Hybond-N+ membrane (Amersham Pharmacia Biotech). The membrane was hybridized with the following probes: a 500 bp *Neu4* cDNA fragment obtained from clone 4156395 by *Pvu*II digestion, an entire *Neu1* cDNA, a 700 bp Neu3 fragment obtained from a cDNA clone by *Pst*I digestion, and an entire cDNA of human β–actin. All probes were

labeled with [^{32}P]-dCTP by random priming using MegaPrime labeling kit (Amersham, Bay d'Urfé, QC). Pre-hybridisation of the blots was performed at 68°C for 30 min in ExpressHybTM (Clontech). The denatured probes were added directly to the pre-hybridisation solution and incubated at 68°C for 1 h. The blots were washed twice for 30 min with 2x SSC, 0.05% SDS at room temperature, once for 40 min with 0.1x SSC, 0.1% SDS at 50°C and exposed to a BioMax film overnight at –80°C.

Neu1 small interfering RNA gene silencing in human skin fibroblasts

SilencerTM pre-designed siRNA ID:8386, ID:8481 and ID:8573 (CAT:16704) targeting exons 1, 2 and 3 of the human Neu1 gene, respectively, were obtained from Ambion (Austin, TX). THP-1 cells were transfected with siRNA using the Nucleofector™ Solution V reagent (Amaxa US) according to the protocol provided by the manufacturer. *GAPDH* siRNA (Ambion) was used as a positive control for the optimization of transfection conditions. The siRNA efficacy was verified in preliminary experiments where THP-1 cells were harvested 24 and 48 hours after transfection with 3 *Neu1* siRNAs, and the sialidase activity was measured with 4 MU-NANA as described above. The most effective *Neu1* siRNA (ID:8481) was selected to perform the suppression experiments. The expression of the Neu1 in the transfected cells was measured on the RNA and protein level by quantitative RT-PCR, Western blotting and enzyme activity assay as described. On average, sialidase activity in the differentiated *Neu1* siRNA-transfected THP-1 cells was reduced more then 4-fold as compared to mock-transfected cells (not shown).

Phagocytosis assay

Differentiation of THP-1 cells was induced by PMA in the presence of either anti- Neu1 rabbit antiserum or control preimmune serum (20 and 100 μg per ml of medium). THP-1 cells transfected with *Neu1* siRNA were differentiated in the presence of PMA only. After 4 days cells seeded on cover slips were incubated within 3 hours with fluorescein-labeled *E. coli* (K- 12 strain), bioparticles (Molecular

Probe, Eugene, OR) in a ratio of 30 particles per one cell, washed 3 times with cold PBS, fixed with 4% paraformaldehyde for 1 h, permeabilized with 0.5% Triton X-100, and washed twice with 0.05% Tween-20 in PBS. Cells were counterstained with 3 μM DAPI and washed again with 0.05% Tween- 20 in PBS. Slides were studied on the Nikon Eclipse E6000 direct epifluorescence microscope.

Measurement of IL and TNF-α Production in Culture Supernatants by Enzyme-linked Immunosorbent Assay

In order to determine the potential effect of anti-Neu1 serum on the secretion of IL-6, IL-10, IL-12, IL-15, IL-1b and TNFa, THP-1 cells cultured in a six-well plate ($3x10^6$ per well) were stimulated with PMA (20 ng/ml) in the absence and in the presence of rabbit pre-immune serum (100 μg/ml) or rabbit antiserum raised against the human Neu1 (20 and 100 μg/ml of medium). After 96-hr incubation, the differentiated cells were further stimulated for 12 hrs by ionomycin (Sigma; 1 μg/ml) and PMA (20 ng/ml) also in the absence and in the presence of rabbit pre-immune serum or rabbit anti-Neu1 antiserum. The concentration of IL and TNF-α in the culture medium was measured by enzyme-linked immunosorbent assay at Chemicon International Inc. (Temecula CA).

RESULTS

Neu1 expression is increased during PMA-induced differentiation of monocytic cell line THP-1

Previous studies defined a monocytic cell line THP-1 from the peripheral blood of a patient with acute monocytic leukemia (14). Being induced by 12-O-tetradecanoylphorbol-13-acetate (PMA) these cells differentiate into adherent cells having morphological, biochemical and physiological characteristics of macrophages and expressing the characteristic macrophage cell surface phenotypic markers, such as CD14+ and CD206+ (28,29). THP-1 cells have several advantages over primary human monocytes (higher transfection efficiency, homogeneity, accessibility) that make them a reliable model to study the mechanism of monocytic differentiation. We

found that during the PMA-induced differentiation, the sialidase activity measured at acidic pH 4.2 with the synthetic fluorescent substrate 2`-(4-methylumbelliferyl)−α−D-Nacetylneuraminic acid (4 MU-NANA) was increased at least 4-fold whereas the levels of other lysosomal enzymes and proteins (cathepsin A, β-galactosidase, β-hexosaminidase, LAMP-2), or of cytosolic enzyme GAPDH were not changed (Fig. 1 A).

The observed increase of the endogeneous sialidase activity could be caused by upregulation of any of 4 previously described human sialidases: lysosomal (Neu1, gene *NEU1* and Neu4, gene *NEU4*), cytoplasmic (Neu2, gene *NEU2*) and plasma membrane (Neu3, gene *NEU3*). Since the cytosolic sialidase Neu2 is mostly active at neutral pH (30), and the sialidase activity measured with 4 MU-NANA at pH 7.5 was not increased during the differentiation of THP-1 cells (Fig. 1 A) we could exclude this enzyme. Three remaining enzymes, Neu1, Neu3 and Neu4 all have acidic pH optimum but they differ from each other in substrate specificity: Neu1 shows the highest activity against oligosacharides and short glycopeptides (24) whereas Neu3 and Neu4 are equally active against α2-3-sialylated oligosaccharides, glycopeptides and gangliosides (25,31,32). Sialidase activity of THP-1 cells measured against sialylated glycolipids (mixed bovine gangliosides) was slightly reduced during differentiation (1.1±0.2 versus 1.7±0.3 nmol/h per mg of total protein, Fig. 1 A) suggesting that the increase of sialidase activity is caused by the upregulation of the lysosomal sialidase *Neu1*. In addition, both quantitative RT-PCR and Northern blots showed that during the differentiation the *Neu1* mRNA was increased at least 5-times (Fig. 1 B and C), whereas both *Neu3* and *Neu4* mRNA were decreased 3 to 4-fold according to the RT-PCR data (Fig. 1 B). Neu2 expression could not be detected in the THP-1 monocytes as well as in the THP-1-derived macrophages neither by RT-PCR nor by Northern blots. Western blots using the polyclonal anti-Neu1 and anti-Neu3 antibodies confirmed up-regulation of *Neu1* and downregulation of *Neu3* at the protein level (Fig. 1 D). Altogether our data showed that the differentiation of THP-1 cells into macrophages results in significant and specific induction of Neu1.

Promoter activity of the *NEU1* gene is increased during the differentiation of THP-1 cells

The transcriptional activity of the *NEU1* gene promoter was studied by deletion mapping. Five restriction fragments of the *NEU1* gene 5'-flanking region were cloned into a pBLCAT6 vector (15) in front of a reporter, chloramphenicol acetyltransferase (CAT) gene and expressed in COS-7 cells (Fig. 2 A). The transfection efficiency was measured using pCMV-β-gal vector containing SV-40 promoter-driven *lacZ* reporter gene. CAT activity in the homogenates of the cells transfected with the reporter constructs was compared to that of the cells transfected with the promoter-free pBLCAT6 vector (15). We found that the shortest fragment with a detectable promoter activity was extending 316 bp upstream of the ATG initiation codon (Fig. 2 B). Truncation of this fragment to 176 bp almost completely abolished the promoter activity. The highest promoter activity was associated with a 542-bp fragment. Extension of this fragment to 936 bp and further to 1598 bp decreased the promoter activity suggesting that potential elements for down-regulation of the gene expression could be present between the nucleotides -542 and -1598 (Fig. 2 B).

We further tested whether the promoter activity of the *NEU1* gene was induced during the differentiation of THP-1 cells into macrophages. THP-1 cells were transfected with the above reporter constructs and 24 hours later their differentiation was induced by adding PMA to the culture medium. We found that 24 hours after the induction of cells with PMA the CAT activity was significantly increased for all constructs, the highest effect (8-fold increase) being achieved for the reporter construct, pBL-NEU1CAT3 containing the 542-bp promoter fragment (Fig. 2 C). For this construct the effect was observed as early as 4 hours after the induction and lasted for at least 4 days (Fig. 2 D). PMA also induced the expression of the reporter gene in COS-7 cells, but the effect was significantly lower then that in THP-1 cells (Fig.2 B).

Interestingly, the analysis of the sequence of the *Neu1* promoter with a TRANSFAC 4.0 software that predicts regulatory elements (33) revealed that the fragment between the nucleotides-316 and -542 contains several conserved sequence motifs for binding promoter-selective transcription factor (Sp-1), activator proteins 1 and 2 (AP-1 and AP-2), hematopoietic transcription factor (E2F-1), adenosin 3', 5'-cyclic monophosphate-regulator element binding factor (CREB-2), and erythroid transcription factors (GATA-1 and NF-E2) (Supplemental Fig. 1) suggesting that some of these factors may be involved in the regulation of the *NEU1* gene transcription.

Indeed, when THP-1 or COS-7 cells transfected with the pBL-NEU1CAT3 reporter construct were treated with a potent AP-1 activator, tumor necrosis factor-α (TNF-α), which increases the synthesis of both *c-Fos* and *c-Jun* proteins in many cell types (34-36) we found a significant induction of the CAT activity (Fig. 3). A detectable increase of the CAT activity was also caused by another AP-1 activator, PDGF-AB (not shown). In all cells the effects of TNF-α PDGFAB or PMA could be blocked by pretreatment of the cells with curcumin and a thiol antioxidant Nacetylcystein (NAC), which inhibit *c-Fos* or *c-Jun* expression (34-36) (Fig. 3).

Neu1 and cathepsin A are targeted to the cell surface during the differentiation of THP-1 cells and primary human monocytes

Intracellular localization of Neu1 in the proliferating THP-1 cells and the THP-1-derived macrophages was studied by confocal immunomicroscopy. In the non-differentiated cells, Neu1 showed a cytoplasmic punctuate staining pattern co-localizing with the lysosomal marker LAMP-2 (Fig. 4 A, Day 0). 24 h after the induction with PMA, the intensity of anti-Neu1 staining was significantly increased and its pattern changed. The cells clearly showed 2 pools of Neu1, one intracellular co-localizing with LAMP-2 and other, peripheral (Fig. 4 A, Day 1). Three days later when THP-1 cells showed morphological characteristics of macrophages, the majority of Neu1 was found on the periphery of the cell, whereas LAMP-2 still had the intracellular localization (Fig. 4 A, Day 4).

To confirm the presence of Neu1 on the surface of the differentiating cells, we performed a double-staining of the non-permeabilized cells with anti-Neu1 antibodies and antibodies against the cell surface mannose receptor, CD206. The proliferating cells did not show any staining for both antigens (Fig. 4 B, Day 0), whereas on the 1st and the 4th days of differentiation the cells showed high expression of both Neu1 and mannose receptor on their surface (Fig. 4 B). Similar results were also obtained for another surface marker, CD14 (not shown) and in the isolated human primary monocytes and the monocyte-derived macrophages (Fig. 4 C and D).

In the lysosome, Neu1 is a component of a multienzyme complex, which also contains the lysosomal carboxypeptidase A (also called cathepsin A or protective protein), ß-galactosidase and N-acetylgalactosamine-6-sulfate sulfatase [reviewed in (4,5)]. If the complex is disrupted, Neu1 loses its enzymatic activity and is rapidly degraded by the lysosomal proteases (6,27). Using confocal microscopy, we showed that during the differentiation of THP-1 cells and isolated monocytes, a significant part of cathepsin A is also targeted to the cell surface. In the non-differentiated THP-1 cells as well as in the primary human monocytes cathepsin A is found only in the lysosomes, co-localizing with LAMP-2 (Fig. 5 A and B, Day 0) and Neu1 (not shown). Starting from the first day of the differentiation and in the mature macrophages, cathepsin A is partially found on the cell periphery where it colocalizes with the mannose receptor (Fig. 5 A, Day 1 and Day 4, Fig. 5 B, Day 7) and Neu1 (not shown).

The translocation of Neu1 and cathepsin A from the lysosomes to the cell surface during the differentiation of monocytes and THP-1 cells was also consistent with the results of enzyme assays. To measure the enzyme activities on the cell surface, intact THP-1 cells or THP-1-derived macrophages were incubated in an isotonic buffer containing the enzyme substrate. To measure the total enzyme activity, the assay was performed in the presence of 0.3% Triton X-100. In the non-differentiated THP-1 cells, the majority of Neu1 was found inside the cells since the activity was ~5-fold higher for the lysed than for the intact cells (Fig. 6A, Neu1). In contrast, in the THP-1-derived macrophages, the majority of Neu1 was present on the

outer cell surface because the activity was increased only by 25% after lysing the cells with Triton X-100. During the differentiation the total sialidase activity assayed in the cell homogenate increased 3.1-fold whereas the sialidase activity on the surface increased 13-fold. In contrast, the activity of a control lysosomal enzyme, N-acetyl-β-hexosaminidase, was similar in the differentiated and non-differentiated cells and in both cases ~77% of the enzyme was not accessible for the substrate in the absence of detergent (Fig. 6A) showing that the ratio of the disrupted and intact cells was similar for macrophages and monocytes. We did not observe an increase of the total cathepsin A activity during the differentiation, however, 70% of the activity in macrophages was detected on the cell surface as opposed to only 25% of the activity in the non-differentiated THP-1 cells (Fig. 6A). Interestingly, neither total activity nor the distributions of the third component of the multienzyme lysosomal complex, β-galactosidase were changed during the differentiation.

Finally, proteins on the surface of THP-1 cells and the THP-1-derived macrophages were biotinylated and affinity purified on a Streptavidin-agarose column. Then, 20 μg of protein from the original cell lysate and the Streptavidin-agarose column flow-through and 5 μg of the biotinylated proteins from the column eluate were analyzed by Western blot using the polyclonal anti-Neu1 antibodies and the antibodies against the 32 kDa peptide chain of cathepsin A (Figure 6B). In the non-differentiated THP-1 cells both Neu1 and cathepsin A were found almost exclusively in the flow-through fraction containing unlabeled intracellular proteins, whereas in the THP-1-derived macrophages a significant percent of these enzymes was biotinylated, suggesting that they are present on the cell surface (Figure 6B).

The differentiation of THP-1 cells induces fusion of the lysosome (endosome)-derived vesicles with the cell surface

Although enzymatically non-active precursors of Neu1 and cathepsin A are activated only in the lysosome (27,37), both enzymes found on the cell surface in macrophages have catalytic activity suggesting that they are first targeted to the lysosome and later to the cell surface possibly in the result of a fusion of the

lysosomal membrane with the plasma membrane. To prove that such fusion happens during the differentiation we have loaded the lysosomes of THP-1 cells with fluorescent dextran and followed its distribution in the cell. In the non-differentiated THP-1 cells the labeled internalized dextran was retained in the endosomal-lysosomal compartment for at least 7 days showing co-localization with the lysosomal marker LAMP-2 (Fig.7, Day 0). In contrast, 12 hours after the induction of the cells with PMA as well as 4 days after, in the fully differentiated THP-1-derived macrophages, the internalized dextran was partially found in the LAMP-2 negative vesicles (which appeared to be migrating to the plasma membrane), on the cell surface and outside the cells while some dextran was still localized in the lysosomes (Fig. 7, Day 1 and Day 4). On the other hand, the differentiation did not increase the secretion of soluble lysosomal enzymes (not shown), suggesting that the vesicles that fuse with the cell surface may lack soluble lysosomal content.

Neu1 colocalizes with MHC II and with the internalized antigens during the differentiation of THP-1

In the differentiated macrophages the internalized antigens are proteolytically digested in the late endosomal-lysosomal compartment and the resulting peptides bind MHC II proteins to be further targeted to the cell surface as the antigenic peptide-MHC II complexes, pMHC [reviewed in (38,39)]. Majority of MHC II is found in the late endosomes, lysosomes and multivesicular bodies (MVBs) collectively called MHC II-enriched compartments (MIICs), although a distinct pMHC loading compartments were also isolated from the antigen-presenting cells and called the class II containing vesicles (CIIV) (40,41). We found that during the differentiation of THP-1 cells and in the THP-1-derived macrophages, Neu1 colocalizes with MIICs. In the non-differentiated cells, the anti-MHC II antibodies only partially colocalized with the anti-Neu1 or the anti-cathepsin A antibodies as well as with the antibodies against the internalized antigen, horseradish peroxidase (HRP) (Fig. 8, Day 0).

However 12 hours after the PMA induction MHC II positive organelles, which also contained the majority of Neu1 and HRP were clearly distinct from the LAMP-2-positive lysosomes (Fig. 8, Day 1). In the fully differentiated macrophages the majority of Neu1, MHC II and anti-HRP immunoreactivity was found on the cell surface (Fig 8, Day 4).

Suppression of the Neu1 expression in the THP-1 cells alters their functional capacity

In order to understand whether upregulation of the Neu1 expression and its targeting to the cell surface is important for the primary function of macrophages we have used siRNA to inhibit the expression of Neu1 in THP-1 cells. The transfected cells which had ~4-fold reduced level of sialidase activity were still capable of PMA-induced differentiation, but showed a significantly reduced ability to ingest the bacteria. When the normal THP-1-derived macrophages were incubated for 3 hours with the fluorescein-labeled *E. coli* bioparticles their ingestion index (percentage of macrophages with phagocytized particles multiplied on the average number of particles phagocytized per ingested macrophage) was ~15.5. For the siRNA transfected cells this number was reduced to ~3.1 (Figure 9). Similar effect on the ingestion capacity was observed when differentiated THP-1 cells were incubated in the presence of anti-Neu1 antibodies (ingestion index ~2.9), whereas the ingestion index of 12.2 for the cells differentiated in the presence of similar amount of pre-immune rabbit serum did not statistically differ from the control (Figure 9).

In a similar fashion, we have observed that the treatment of THP-1-derived macrophages with the anti-Neu1 antibodies significantly reduced their ability to produce certain cytokines. As before, the cells were differentiated in the presence of the anti-Neu1 antiserum or the preimmune rabbit serum and then stimulated for 12 hrs by ionomycin also in the presence of the anti-Neu1 antiserum or the preimmune rabbit serum. We found that the anti-Neu1 antibodies but not the pre-immune serum have inhibited ionomycin induced production of IL-1b, IL-6 and TNF-α, whereas the

changes in the production of IL-10 and IL-12 (p40) were not statistically significant (Figure 10).

DISCUSSION

The endogenous sialidase activity significantly increases during activation in majority of immune cells including T cells, B cells, and monocytes, whereas sialylation of some of their surface molecules decreases (10,42-45). In particular, the reduction of sialylation was reported for MHC class I, required to render T cells responsive to antigen presenting cells (43), G_{M3}-ganglioside, which modulates Ca^{2+} immobilization and regulates IL-4 production in T cells (11) and monocyte receptor for hyaluronic acid, CD44 (44,45). In the activated B cells the induction of the sialidase activity is important to convert so-called, vitamin D3-binding protein into a macrophage-activating factor (MAF) (46). Indirect evidences suggested the involvement in these processes of Neu1, an ubiquitously expressed lysosomal sialidase responsible for the catabolism of glycoconjugates. *In vivo*, SM/J or SM/B10 mice strains with the missense mutation in the *NEU1* gene which reduces the enzyme activity to ~30% of normal (47) have an impaired activation of macrophages while their lymphocytes fail to synthesize IL-4, show low response for antigen presenting cells and do not produce MAF (10,42,46). In human sialidosis patients Neu1 deficiency may result in reduced capacity of immune cells to produce cytokines and antibodies leading to partial immunodeficiency and may account for frequent pulmonary infections (G. Mitchell, personal communication). We have provided a direct evidence that Neu1 message and protein are induced several fold during the differentiation of circulating human monocytes into professional antigen-presenting cells (APC) such as macrophages (13) or dendritic cells, (Stamatos N., manuscript in preparation). In this study we investigated the mechanisms controlling the expression of the *NEU1* gene and found that the transcriptional activity of the *NEU1* gene promoter was significantly increased during the PMA-induced differentiation of

monocytic cells into macrophages. The highest level of induction was observed for a 550-bp fragment of the *NEU1* gene promoter containing several potential sequence motifs for binding AP-1, a group of homodimeric and heterodimeric complexes composed of various *Fos* and *June* gene products such as *c-Jun*, *junB*, *junD*, *c-Fos*, *fosB* as well as the fos-related antigens *Fra-1* and *Fra-2* (48-54). All these complexes (also called protooncoproteins) interact with the common binding sites called PMA-responsive elements and mediate gene induction in response to growth factors, cytokines, phorbol esters and a variety of other stimuli including oxidative stress (34-36). In particular, one of AP-1 functions is to mediate inflammatory responses in lymphocytes and macrophages by stimulating cell activation and the expression of cytokines (55). Previously it was shown that pro-inflammatory factors, LPS and TNF-α caused 16-hour MAPK-dependent induction of the endogenous acidic sialidase activity in monocytes (44,45) resulting in desialylation of CD44. In turn, this increased the affinity of monocytes to hyaluronic acid, their adhesion and the TNF-α production (45). Since in both cases the 1.4-1.6-fold increase of the sialidase activity was detected only with 4 MU-NANA but not with mixed bovine gangliosides as a substrate it is conceivable that the effect was caused by the upregulation of *Neu1*. Our experiments directly showed that the promoter activity of Neu1 was potently induced by TNF-α that activates AP-1 expression and reduced by AP-1 inhibitors curcumin and N-acetylcysteine. Although we do not have a direct evidence of the interaction between the Neu1 gene promoter and AP-1 it is tempting to speculate that this protein may be involved in the regulation of the Neu1 expression during the monocyte activation and differentiation.

The fact that Neu1 is specifically induced in professional APCs suggests that this enzyme may be implicated in the antigen presentation. However both MHC class II and I molecules are expressed at the cell surface while Neu1 was found predominantly in the endosomal/lysosomal compartment. Here we show that during the differentiation of monocytes into macrophages the Neu1 is constitutively transported to the plasma membrane along with class II molecules rather then being actively retained in the lysosomes. Using immunocytochemistry we demonstrated

that in the differentiating THP-1 cells, THP-1-derived macrophages as well as in the mature primary macrophages a vast majority of the Neu1 was found on the cell periphery co-localizing with the mannose receptor. In addition, biochemical assays showed that the biotinylation of the cell surface proteins results in the labeling of Neu1 in macrophages but not in the proliferating THP-1 cells and that ~80% of Neu1 in the THP-1-derived macrophages was accessible for the substrate added directly to the cell medium confirming that it is localized on the cell surface. Similar observations were made for the lysosomal carboxypeptidase, cathepsin A, whose interaction with Neu1 is essential for the activation of Neu1 in the lysosomes.

Both Neu1 and cathepsin A are sorted from the trans-Golgi network to the endosomal pathway. As the majority of soluble lysosomal lumenal proteins cathepsin A is targeted through the mannose-6-phosphate receptor pathway (56), whereas the intracellular trafficking of Neu1 is mediated by the adaptor proteins through a Tyr-containing motif in its C-terminus (9). In the lysosome the two proteins undergo activation. The activation of cathepsin A involves its processing by a lysosomal endopeptidase which removes a 2-kDa "linker" polypeptide between the two polypeptide chains of the mature enzyme (57). The exact molecular mechanism that underlies the activation of Neu1 is unknown but most likely it involves a conformational change of the Neu1 polypeptide after the formation of the complex with cathepsin A. We speculate that in the differentiating cells both enzymes are first targeted to the endosomal/lysosomal compartment, where they gain enzymatic activity and then are sorted to the vesicles destined to the plasma membrane. The existence of such lysosome-to-plasma membrane transport pathway has been well documented for other professional APCs, dendritic cells where it plays a crucial role in targeting the peptide-MHC II complexes (pMHCs) to the cell surface (58). Upon receipt of a maturation stimulus (such as lipopolysacharide) the internalized antigens are processed in the endosomes and pMHCs are formed. Several hours later they appear in the LAMP-negative compartments termed CIIV, which are the intermediates in the transfer of pMHCs to the plasma membranes (58). The concentration of pMHCs in CIIV reaches a maximum 12 hours after the

lipopolysacharide treatment and after 12 more hours the majority of complexes is already transferred to the cell surface. The described targeting of pMHCs in dendritic cells resembles the targeting of Neu1 and cathepsin A in differentiating monocytes and macrophages. We found that 12 to 24 hours after receiving the differentiation stimulus the lysosomal-endosomal apparatus of monocytes undergoes a dramatic rearrangement. The MHC II-positive and Neu1-positive compartments are segregated from the LAMP-2 positive lysosomes and late endosomes, relocated closer to the cell surface and fused with the plasma membranes. The same vesicles are stained positively for the internalized antigen HRP or fluorescent dextran, which in the proliferating monocytic cells are normally stored for at least several days in LAMP-2-positive endosomal/lysosomal compartment. In the differentiating cells the dextran molecules and HRP peptides (probably in the form of pMHCs) were gradually accumulated on the cell plasma membrane colocalizing at least in part with Neu1 and cathepsin A.

Most importantly, the suppression of Neu1 expression in the THP-1-derived macrophages by siRNA or their treatment with anti-Neu1 antibodies significantly reduced functional capacity of the cells, such as the ability to engulf bacteria or to produce cytokines, suggesting that the upregulation of the Neu1 expression is important for activation of macrophages and establishing the link between Neu1 and cellular immune response. Further experiments should clarify if the differentiation-induced translocation of the lysosomal sialidase, Neu1 and the lysosomal carboxypeptidase, cathepsin A to the plasma membrane is also important for other specific functions of the macrophages and, in particular, for the antigen presentation. Optimal sialylation of the cell surface glycoproteins may also be important for the interaction of macrophages with other cells, e.g., with T, B and NK cells via sialic acid-binding immunoglobulin-like lectins [reviewed in (59)]. Fully differentiated macrophages are present in all body tissues (microglial cells in the CNS, Kupffer cells in liver, etc.). Therefore, it is quite conceivable that if upregulation of the *NEU1* gene is functionally important for macrophage differentiation, then the primary or

secondary deficiency of the Neu1 activity may affect functions of all monocyte-derived cells and cause defects in multiple tissues and organs.

FOOTNOTES

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REFERENCES

1. Pshezhetsky, A. V., Richard, C., Michaud, L., Igdoura, S., Wang, S., Elsliger, M. A., Qu, J., Leclerc, D., Gravel, R., Dallaire, L., and Potier, M. (1997) *Nat.Genet.* **15,** 316-320
2. Bonten, E., van der, S. A., Fornerod, M., Grosveld, G., and d'Azzo, A. (1996) *Genes Dev* **10,** 3156- 3169

3. Milner, C. M., Smith, S. V., Carrillo, M. B., Taylor, G. L., Hollinshead, M., and Campbell, R. D. (1997) *J Biol Chem.* **272,** 4549-4558

4. d'Azzo, A., Andria, G., Strisciuglio, G., and Galjaard, H. (2001) Galactosialidosis. In Scriver, C. R., Beaudet, A. L., Sly, W. S., and Valle, D., editors. *Metabolic and Molecular Bases of Inherited Disease*, McGraw-Hill, New York

5. Pshezhetsky, A. V. and Ashmarina, M. (2001) *Prog.Nucleic Acid Res.Mol.Biol.* **69,** 81-1146. van der Horst, G. T., Galjart, N. J., d'Azzo, A., Galjaard, H., and Verheijen, F. W. (1989) *J Biol Chem.* **264,** 1317-1322

7. Thomas, G. H. and Beaudet, A. L. (2001) Disorders of glycoprotein degradation and structure: α-mannosidosis, β-mannosidosis, fucosidosis, sialidosis, aspartylglucosaminuria, and carbohydratedeficient glycoprotein syndrome. In Scriver, C. R., Beaudet, A. L., Sly, W. S., and Valle, D., editors. *The Metabolic and Molecular Bases of Inherited Disease*, McGraw-Hill, New York

8. Seyrantepe, V., Poupetova, H., Froissart, R., Zabot, M. T., Maire, I., and Pshezhetsky, A. V. (2003) *Hum.Mutat.* **22,** 343-352

9. Lukong, K. E., Seyrantepe, V., Landry, K., Trudel, S., Ahmad, A., Gahl, W. A., Lefrancois, S., Morales, C. R., and Pshezhetsky, A. V. (2001) *J.Biol.Chem.* **276,** 46172-46181

10. Chen, X. P., Enioutina, E. Y., and Daynes, R. A. (1997) *J.Immunol.* **158,** 3070-3080

11. Chen, X. P., Ding, X., and Daynes, R. A. (2000) *Cytokine* **12,** 972-985

12. Wang, P., Zhang, J., Bian, H., Wu, P., Kuvelkar, R., Kung, T. T., Crawley, Y., Egan, R. W., and Billah, M. M. (2004) *Biochem.J.* **380,** 425-433

13. Stamatos NM, L. F. N. X. L. K. C. A. W. L. P. A. (2005) *FEBS J* **272,** 2545-2556

14. Tsuchiya, S., Yamabe, M., Yamaguchi, Y., Kobayashi, Y., Konno, T., and Tada, K. (1980) *Int.J.Cancer* **26,** 171-176

15. Boshart, M., Kluppel, M., Schmidt, A., Schutz, G., and Luckow, B. (1992) *Gene* **110,** 129-130

16. Alam, J. and Cook, J. L. (1990) *Anal.Biochem.* **188,** 245-254

17. Bradford, M. M. (1976) *Anal.Biochem.* **72,** 248-254

18. Potier, M., Mameli, L., Belisle, M., Dallaire, L., and Melancon, S. B. (1979) *Anal.Biochem.* **94,** 287-296

19. Rome, L. H., Garvin, A. J., Allietta, M. M., and Neufeld, E. F. (1979) *Cell* **17,** 143-153

20. Tranchemontagne, J., Michaud, L., and Potier, M. (1990) *Biochem.Biophys.Res Commun.* **168,** 22-29

21. Bergmeyer, H., Gawhen, K., and Grassl, M. (1974) Phosphatase. In Bergmeyer, H. V., editor. *Methods of Enzymatic Analysis*, Verlag-Chemie, Weinheim, Federal Republic of Germany

22. Schmidt, E. (1974) Glutamate dehydrogenase. In Bergmeyer, H. V., editor. *Methods of Enzymatic Analysis*, Verlag-Chemie, Weinheim, Federal Replubic of Germany

23. Ryazanov, A. G., Ashmarina, L. I., and Muronetz, V. I. (1988) *Eur.J.Biochem.* **171,** 301-305

24. Miyagi, T., Hata, K., Hasegawa, A., and Aoyagi, T. (1993) *Glycoconj.J.* **10,** 45-49

25. Schneider-Jakob, H. R. and Cantz, M. (1991) *Biol Chem.Hoppe Seyler* **372,** 443-450

26. Manzi, A. E. (1994) Preparation and analysis of glycoconjugates. In Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., editors. *Current Protocols in Molecular Biology*, J Wiley & Sons,

27. Vinogradova, M. V., Michaud, L., Mezentsev, A. V., Lukong, K. E., El Alfy, M., Morales, C. R., Potier, M., and Pshezhetsky, A. V. (1998) *Biochem.J.* **330 (Pt 2),** 641-650

28. Tsuchiya, S., Kobayashi, Y., Goto, Y., Okumura, H., Nakae, S., Konno, T., and Tada, K. (1982) *Cancer Res.* **42,** 1530-1536

29. Auwerx, J. (1991) *Experientia* **47,** 22-31

30. Monti, E., Preti, A., Nesti, C., Ballabio, A., and Borsani, G. (1999) *Glycobiology* **9,** 1313-1321

31. Li, S. C., Li, Y. T., Moriya, S., and Miyagi, T. (2001) *Biochem.J.* **360,** 233-237

32. Seyrantepe, V., Landry, K., Trudel, S., Hassan, J. A., Morales, C. R., and Pshezhetsky, A. V. (2004) *J.Biol.Chem.* **279,** 37021-37029

33. Wingender, E., Chen, X., Fricke, E., Geffers, R., Hehl, R., Liebich, I., Krull, M., Matys, V., Michael, H., Ohnhauser, R., Pruss, M., Schacherer, F., Thiele, S., and Urbach, S. (2001) *Nucleic Acids Res.* **29,** 281-283

34. Haliday, E. M., Ramesha, C. S., and Ringold, G. (1991) *EMBO J.* **10,** 109-115

35. Hanazawa, S., Takeshita, A., Amano, S., Semba, T., Nirazuka, T., Katoh, H., and Kitano, S. (1993) *J.Biol.Chem.* **268,** 9526-9532

36. Lo, Y. Y. and Cruz, T. F. (1995) *J.Biol.Chem.* **270,** 11727-11730

37. Galjart, N. J., Morreau, H., Willemsen, R., Gillemans, N., Bonten, E. J., and d'Azzo, A. (1991) *J Biol Chem.* **266,** 14754-14762

38. Bryant, P. and Ploegh, H. (2004) *Curr.Opin.Immunol.* **16,** 96-102

39. Mellman, I. and Steinman, R. M. (2001) *Cell* **106,** 255-258

40. Amigorena, S., Webster, P., Drake, J., Newcomb, J., Cresswell, P., and Mellman, I. (1995) *J.Exp.Med.* **181,** 1729-1741

41. Pierre, P., Denzin, L. K., Hammond, C., Drake, J. R., Amigorena, S., Cresswell, P., and Mellman, I. (1996) *Immunity.* **4,** 229-239

42. Landolfi, N. F., Leone, J., Womack, J. E., and Cook, R. G. (1985) *Immunogenetics* **22,** 159-167

43. Landolfi, N. F. and Cook, R. G. (1986) *Mol.Immunol.* **23,** 297-309

44. Katoh, S., Miyagi, T., Taniguchi, H., Matsubara, Y., Kadota, J., Tominaga, A., Kincade, P. W., Matsukura, S., and Kohno, S. (1999) *J.Immunol.* **162,** 5058-5061

45. Gee, K., Kozlowski, M., and Kumar, A. (2003) *J.Biol.Chem.* **278,** 37275-37287

46. Yamamoto, N. and Kumashiro, R. (1993) *J Immunol.* **151,** 2794-2802

47. Rottier, R. J., Bonten, E., and d'Azzo, A. (1998) *Hum.Mol.Genet.* **7,** 313-321

48. Cohen, D. R., Ferreira, P. C., Gentz, R., Franza, B. R., Jr., and Curran, T. (1989) *Genes Dev.* **3,** 173-184

49. Curran, T. and Franza, B. R., Jr. (1988) *Cell* **55,** 395-397

50. Lee, W., Mitchell, P., and Tjian, R. (1987) *Cell* **49,** 741-752

51. Ransone, L. J. and Verma, I. M. (1990) *Annu.Rev.Cell Biol.* **6,** 539-557

52. Rauscher, F. J., III, Cohen, D. R., Curran, T., Bos, T. J., Vogt, P. K., Bohmann, D., Tjian, R., and Franza, B. R., Jr. (1988) *Science* **240,** 1010-1016

53. Rauscher, F. J., III, Sambucetti, L. C., Curran, T., Distel, R. J., and Spiegelman, B. M. (1988) *Cell* **52,** 471-480

54. Schonthal, A., Herrlich, P., Rahmsdorf, H. J., and Ponta, H. (1988) *Cell* **54,** 325-334

55. Tanaka, C., Kamata, H., Takeshita, H., Yagisawa, H., and Hirata, H. (1997) *Biochem.Biophys.Res.Commun.* **232,** 568-573

56. Lukong, K. E., Elsliger, M. A., Mort, J. S., Potier, M., and Pshezhetsky, A. V. (1999) *Biochemistry* **38,** 73-80

57. Bonten, E. J., Galjart, N. J., Willemsen, R., Usmany, M., Vlak, J. M., and d'Azzo, A. (1995) *J Biol Chem.* **270,** 26441-26445

58. Turley, S. J., Inaba, K., Garrett, W. S., Ebersold, M., Unternaehrer, J., Steinman, R. M., and Mellman, I. (2000) *Science* **288,** 522-527

59. Crocker, P. R. (2002) *Curr.Opin.Struct.Biol.* **12,** 609-615

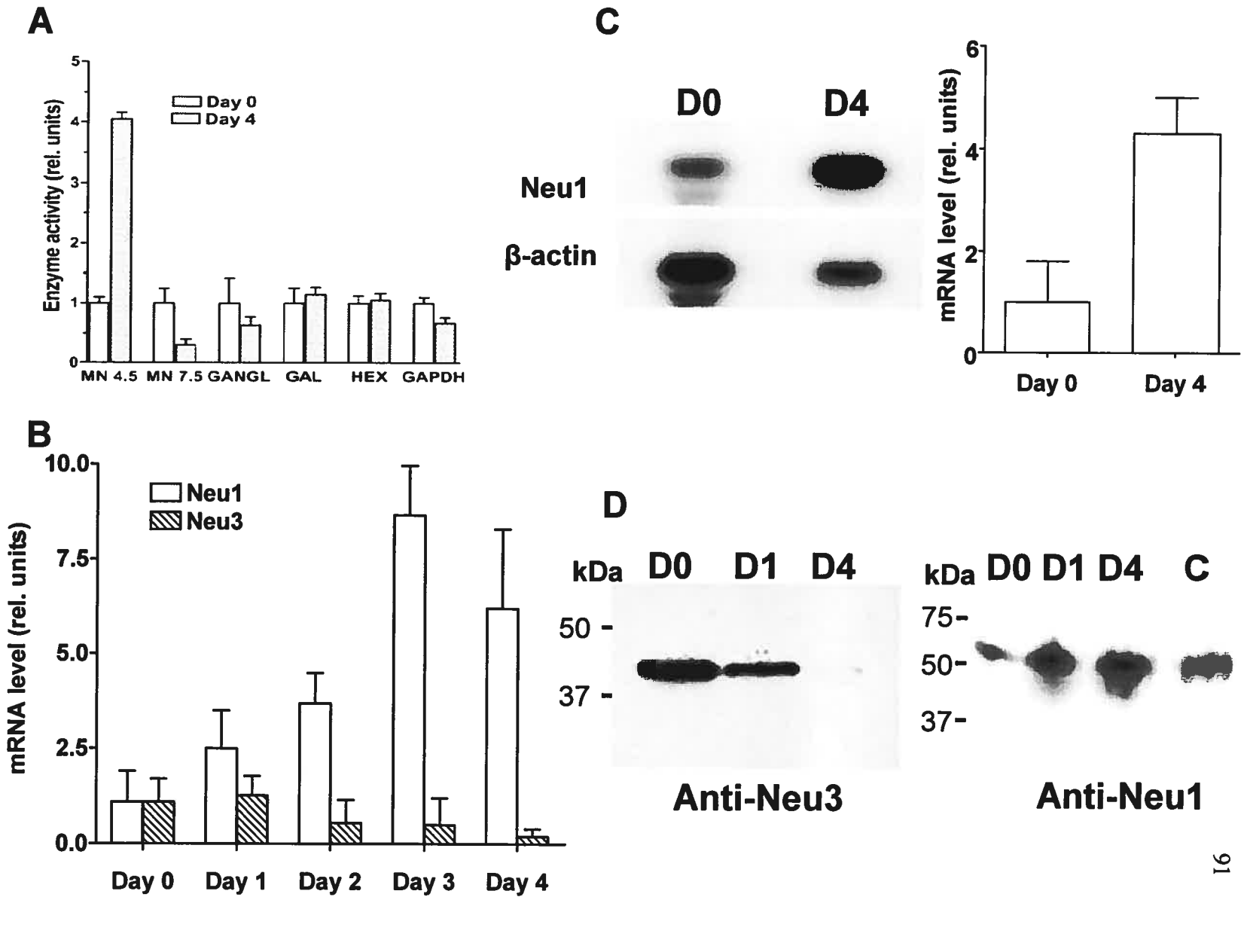

A
Enzyme activity (rel. units)
Day 0
Day 4
MN 4.5
MN 7.5
GANGL
GAL
HEX
GAPDH
B
mRNA level (rel. units)
Neu1
Neu3
Day 0
Day 1
Day 2
Day 3
Day 4
C
D0
D4
Neu1
β-actin
mRNA level (rel. units)
Day 0
Day 4
D
kDa
D0
D1
D4
50
37
Anti-Neu3
kDa
D0
D1
D4
C
75
50
37
Anti-Neu1

Figure 1

Figure 1. Induction of Neu1 during the differentiation of THP-1 cells into macrophages

A. Specific activity of sialidase against the synthetic substrate MU-NANA at pH 4.5 (***MN 4.5***) and pH 7.5 (***MN 7.5***) and gangliosides (***GANGL***), β-galactosidase (***GAL***), β-hexosaminidase (***HEX***) and glyceraldehyde-3-phosphate dehydrogenase (***GAPDH***) measured in total homogenates of THP-1 cells before (***clear bars***) or 4 days after (***shaded bars***) the PMA treatment. Values represent means ± S.D. of triplicate experiments. Data are shown as fractions of the activity measured in undifferentiated THP-1 cells.

B. mRNA levels of Neu1 and Neu3 in THP-1 cells during the PMA-induced differentiation. The mRNA levels quantified by RT-PCR as described in Materials and Methods are presented as a fraction of the level detected in the proliferating THP-1 cells. Values represent means ± S.D. of triplicate independent experiments.

C. Northern blotting of RNA from undifferentiated THP-1 cells (***Day 0***) and the cells treated for 4 days with 20 nM PMA (***Day 4***). The membranes were hybridised with $^{[32]}$P-labelled ***Neu1*** and ***β-actin*** cDNA probes and exposed to a BioMax film (***left***). Images were scanned and relative intensities of the signals from ***Neu1*** and ***β-actin*** probes quantified using a PDQuest software (Bio-Rad) (***right***). Values represent means ± S.D. of triplicate independent experiments.

D. Western blotting of undifferentiated THP-1 cells (***Day 0***) and the cells treated for 1 (***Day 1***) and 4 days (***Day 4***) with 20 nM PMA. Each lane contains 20 μg of total cell lysates. ***C***, positive control, Neu1 purified from human placenta. The immunoblotting was performed with anti-Neu1 and anti-Neu3 rabbit polyclonal antibodies as indicated.

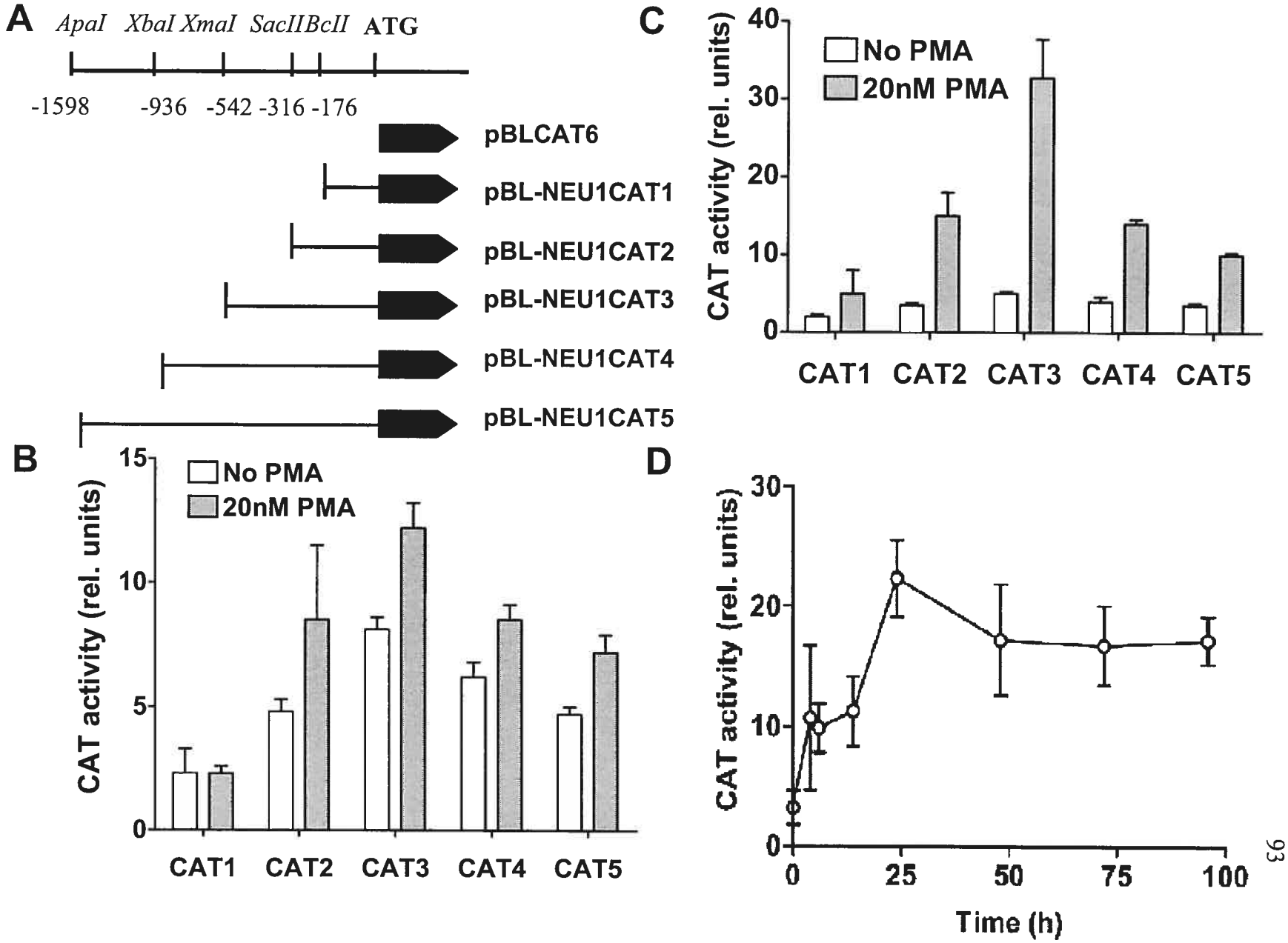

A
ApaI XbaI XmaI SacIIBclI ATG
-1598 -936 -542 -316 -176
pBLCAT6
pBL-NEU1CAT1
pBL-NEU1CAT2
pBL-NEU1CAT3
pBL-NEU1CAT4
pBL-NEU1CAT5
B
CAT activity (rel. units)
No PMA
20nM PMA
CAT1 CAT2 CAT3 CAT4 CAT5
C
CAT activity (rel. units)
No PMA
20nM PMA
CAT1 CAT2 CAT3 CAT4 CAT5
D
CAT activity (rel. units)
Time (h)

Figure 2

Figure 2. Transcriptional activity of the *NEU1* gene promoter and its activation during the differentiation of THP-1 cells

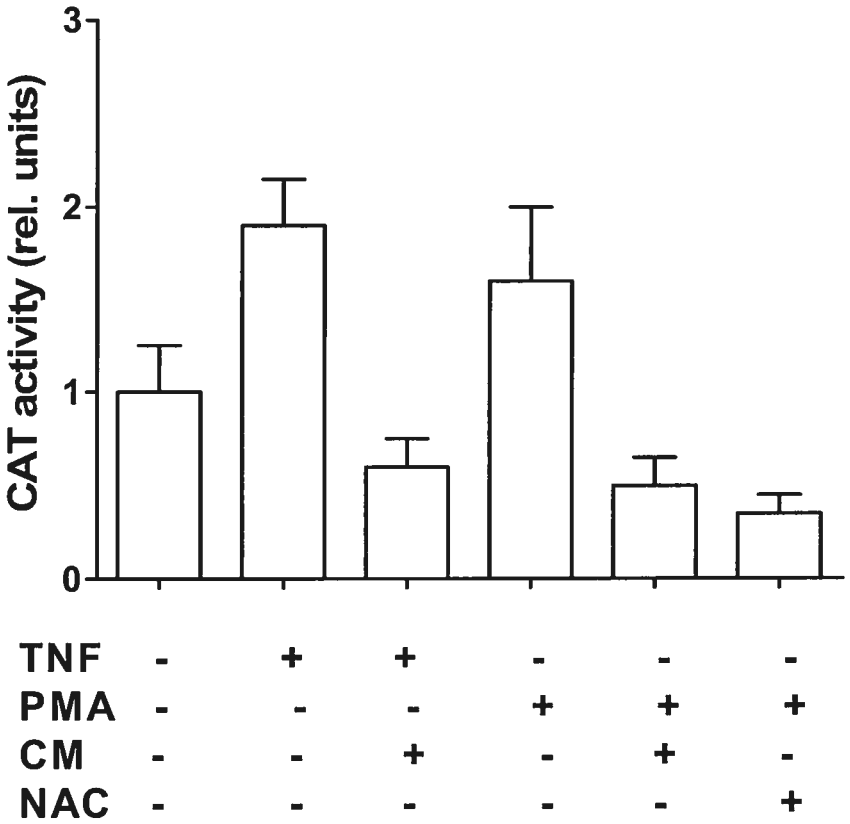
A. Reporter constructs containing the restriction fragments of the 5`-flanking region of the *NEU1* gene cloned into pBLCAT6 vector in front of the reporter *CAT* cDNA.

B. Transcriptional activity of the *NEU1* promoter fragments in COS-7 cells. Twenty-four hours after the transfection cells were incubated in the medium containing 0 or 20 mM of PMA for another 24 h. The CAT activity in cell homogenates normalized for the transfection efficiency is shown as a fraction of the activity measured in the cells transfected with promoter-free pBLCAT6 vector. Values represent means ± S.D. of triplicate independent experiments.

C. Transcriptional activity of the *NEU1* promoter fragments in THP-1 cells. Twenty-four hours after the transfection cells were incubated in the medium containing 0 or 20 mM of PMA for another 24 h. The CAT activity is shown as in panel B.

D. Transcriptional activity of the 542-bp fragment of the *NEU1* gene promoter in THP-1 cells during the PMA-induced differentiation. Twenty-four hours after the transfection with the reporter construct pBL-NEU1CAT3 cells were incubated in the medium containing 20 mM of PMA. At time intervals indicated on the figure the cells were harvested and the CAT activity was measured in cell homogenates. The CAT activity is shown as fraction of the activity measured in the non-treated cells. Values represent means ± S.D. of triplicate independent experiments.

A


CAT activity (rel. units)
3
2
1
0
TNF - + + - - -
PMA - - - + + +
CM - - + - + -
NAC - - - - - +

B

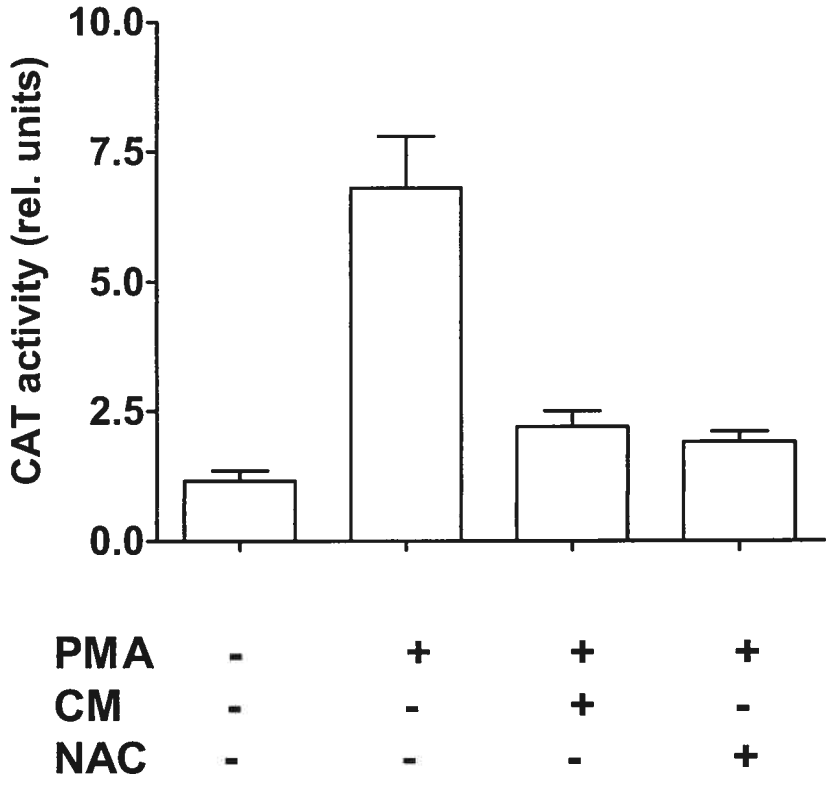

CAT activity (rel. units)
10.0
7.5
5.0
2.5
0.0
PMA - + + +
CM - - + -
NAC - - - +

Figure 3

Figure 3. Effect of TNF-α, NAC and curcumin on *NEU1* promoter-driven CAT expression in COS-7 (A) and THP-1 (B) cells

Twenty-four hours after the transfection with pBL-NEU1CAT3 vector the cells were stimulated for another 24 h with TNF-α (40 ng/ml) or PMA (20 nM) in the presence or absence of curcumin (***CM***, 0.5 μM) and NAC (***NAC***, 20 mM) as indicated on the figure. The CAT activity values normalized for the transfection efficiency are shown as a fraction of the activity measured in the absence of TNF-α or PMA. Values represent means ± S.D. of triplicate independent experiments.

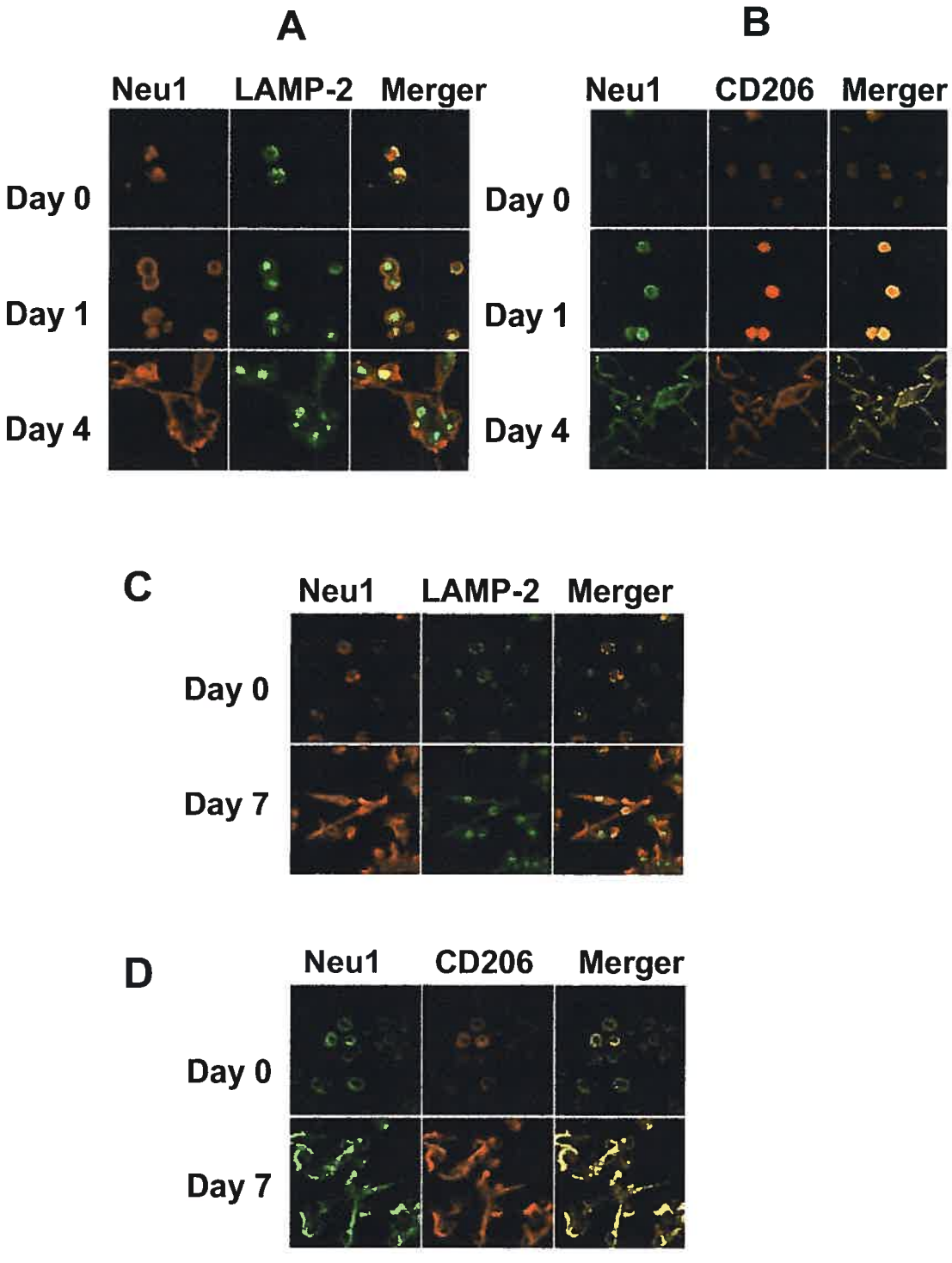

A
Neu1
LAMP-2
Merger
Day 0
Day 1
Day 4
B
Neu1
CD206
Merger
Day 0
Day 1
Day 4
C
Neu1
LAMP-2
Merger
Day 0
Day 7
D
Neu1
CD206
Merger
Day 0
Day 7

Figure 4

Figure 4. Immunohistochemical localization of Neu1 during the differentiation of THP-1 cells (A, B), and primary monocytes (C, D).

After 0, 1, 4 and 7 days of differentiation the cells were fixed, stained with rabbit polyclonal anti-Neu1 antibodies, and counterstained with mouse monoclonal antibodies either against human ***LAMP-2*** or against human mannose receptor (***CD206***) as indicated on the figure. Panels A, C and D show permeabilized cells, panel B nonpermeabilized cells. Panel represent randomly selected typical images obtained in triplicate experiments; from 140 to 200 cells were studied for each condition in each experiment. Slides were examined on a Zeiss LSM510 inverted confocal microscope. Magnification 620x.

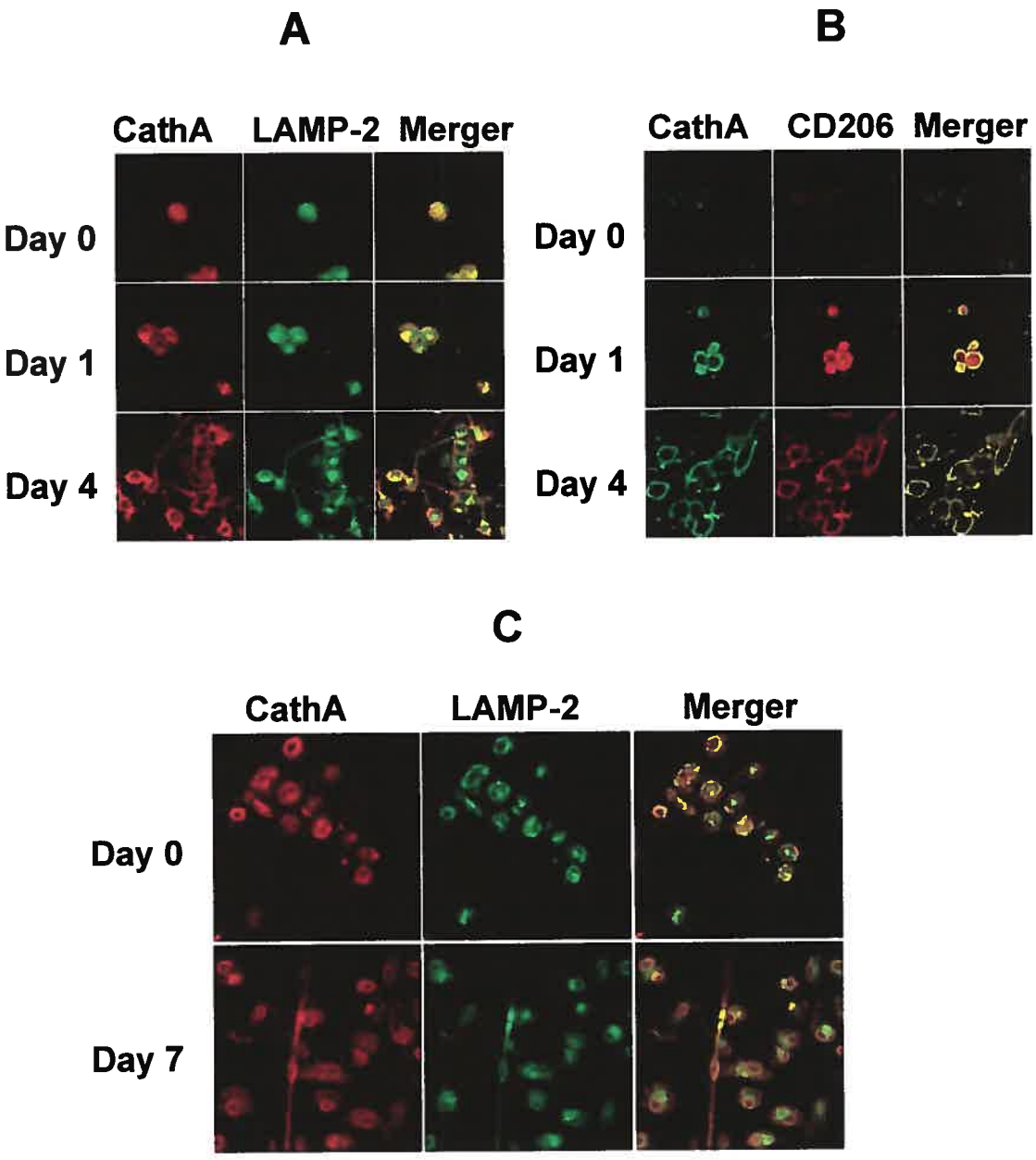

A
CathA
LAMP-2
Merger
Day 0
Day 1
Day 4
B
CathA
CD206
Merger
Day 0
Day 1
Day 4
C
CathA
LAMP-2
Merger
Day 0
Day 7

Figure 5

Figure 5. Immunohistochemical localization of cathepsin A (*CathA*) during the differentiation of THP-1 cells (A, B), and primary monocytes (C)

After 0, 1, 4 and 7 days of differentiation the cells were fixed, stained with rabbit polyclonal anti-cathepsin A antibodies, and counterstained with mouse monoclonal antibodies against human ***LAMP-2*** or against human mannose receptor (***CD206***) as indicated on the figure. Panels A and C show permeabilized cells, panel B, nonpermeabilized cells. Panel represent randomly selected typical images obtained in triplicate experiments; from 140 to 200 cells were studied for each condition in each experiment. Slides were examined on a Zeiss LSM510 inverted confocal microscope. Magnification 620x.

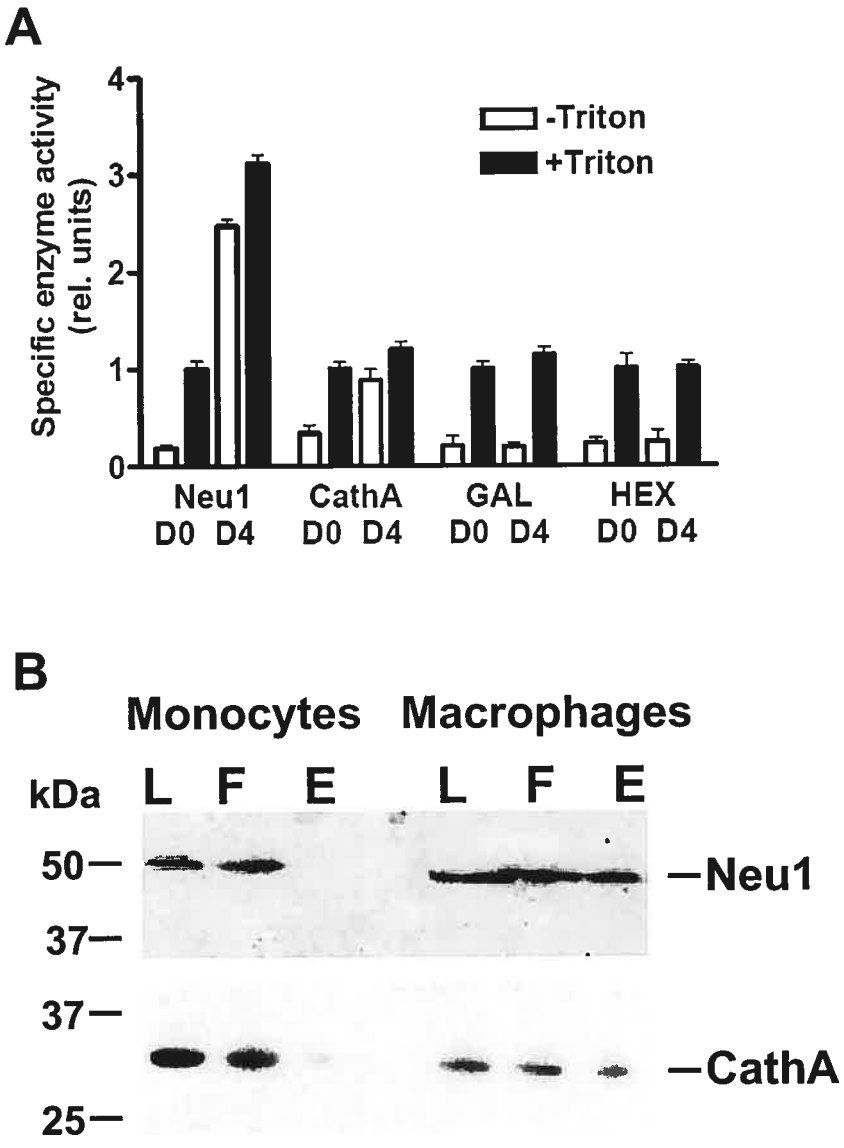

A
Specific enzyme activity (rel. units)
4
3
2
1
0
-Triton
+Triton
Neu1
D0 D4
CathA
D0 D4
GAL
D0 D4
HEX
D0 D4
B
Monocytes
Macrophages
kDa
L F E
L F E
50—
37—
37—
25—
—Neu1
—CathA

Figure 6

Figure 6. Biochemical confirmation of the cells surface localization of Neu1 and cathepsin A in the THP-1-derived macrophages.

A. Enzyme activity on the surface of THP-1 cells and THP-1-derived macrophages

Non-differentiated (***D0***) and differentiated (***D4***) THP-1 cells were incubated in the isotonic buffer solution containing sialidase substrate 4 MU-NANA (***Neu1***), cathepsin A (***CathA***), β-galactosidase (***GAL***) and N-acetyl β-hexosaminidase (***HEX***) substrates in the absence (open bars) or in the presence (black bars) of 0.3% (v/v) Triton X-100 as described. Values represent means ± S.D. of triplicate experiments.

B. Biotinylation of the cell surface pool of Neu1 and cathepsin A in THP-1-derived macrophages

Cell surface proteins of proliferating THP-1 cells and THP-1-derived macrophages were labeled with a BAC-SulfoNHS biotinylation reagent and purified on Streptavidin-agarose column as described in the Materials and Methods. Twenty μg of protein from the original cell lysate (**L**) and Streptavidin-agarose column flow-through (**F**) and 5 μg of protein from the eluate (**E**) were separated on a NuPAGE 4-12% Bis-Tris gel and transfered to a PVDF membrane. The membrane was hybridized with the polyclonal anti-Neu1 antibodies (upper panel) and anti-cathepsin A antibodies (lower panel). The detection was performed using a Roche Western kit. The positions of 32-kDa protein chain of cathepsin A (***CathA***) and Neu1 are shown on the right side of the blot.

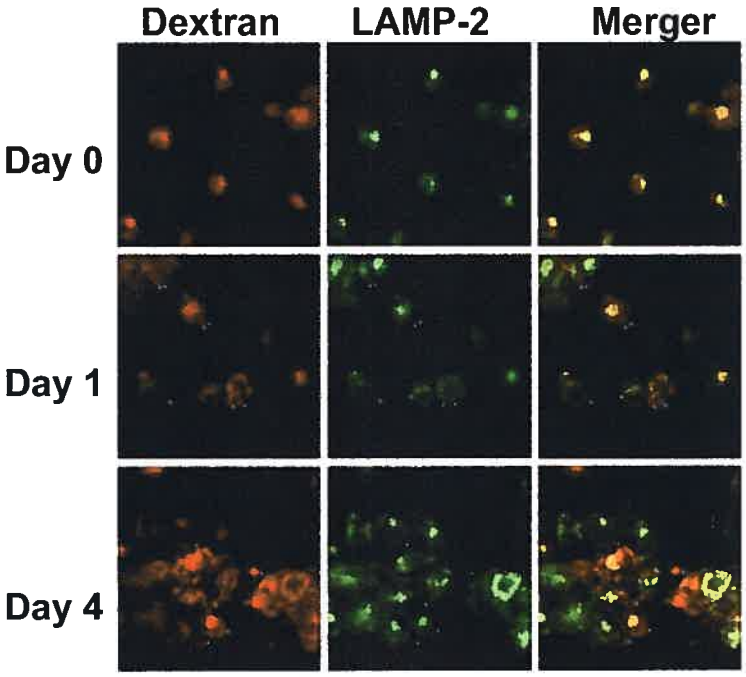


Figure 7

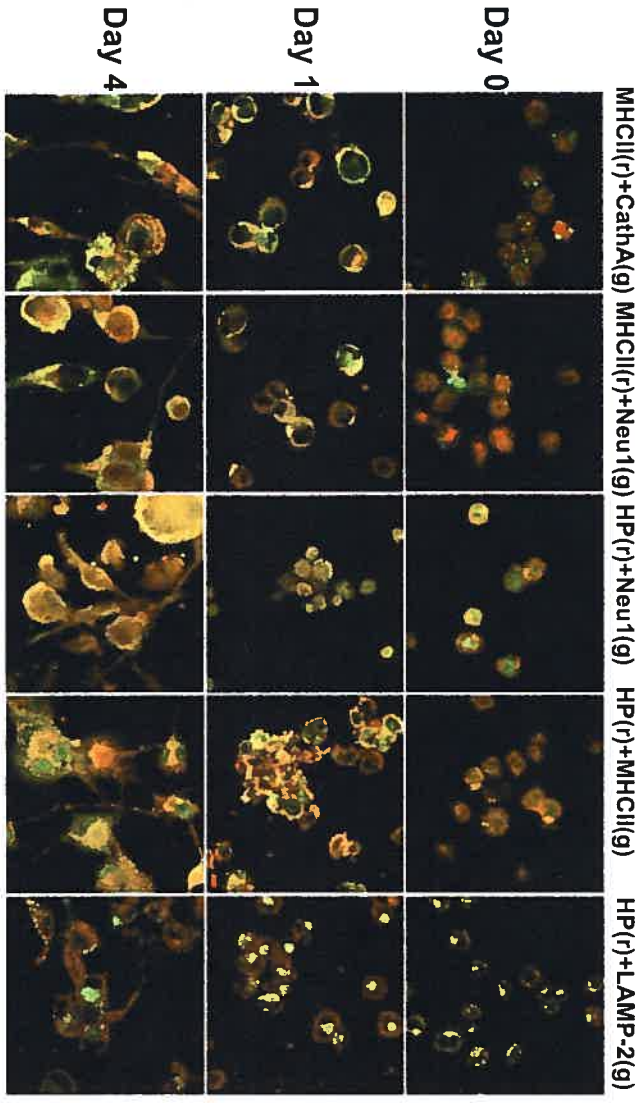


Figure 8

Figure 7. Differentiation of THP-1 cells induces the targeting of internalized Texas Red-labeled dextran to the cell surface.

In nondifferentiated cells (***Day 0***) the labeled dextran is colocalized with the lysosomal marker LAMP-2. In merged images of the cells harvested 12 h after the induction of the cells with PMA (***Day 1***) and of the differentiated cells (***Day 4***) a part of the internalized dextran appears red (***arrowheads***) indicating that the dextran is located in LAMP-2 negative vesicles, (which appeared to be migrating to the plasma membrane), on the cell surface or outside the cells. Panels represent randomly selected typical images obtained in triplicate experiments; ~80 cells were studied for each condition in each experiment.

Figure 8. Immunohistochemical localization of Neu1, cathepsin A, MHC II and the internalized antigen, horseradish peroxidase during the differentiation of THP-1 cells.

Twelve hours after the loading with horseradish peroxidase (HRP) THP-1 cells were treated with PMA as described. After 0 h, 24 h and 4 days of differentiation the cells were fixed, stained with antibodies against ***HRP, LAMP-2, Neu1***, cathepsin A (***CathA***) and ***MHC II*** as indicated on the figure. Panels represent randomly selected typical images obtained in triplicate experiments; ~50 cells were studied for each condition in each experiment. Slides were examined on a Zeiss LSM510 inverted confocal microscope. Magnification 620x.

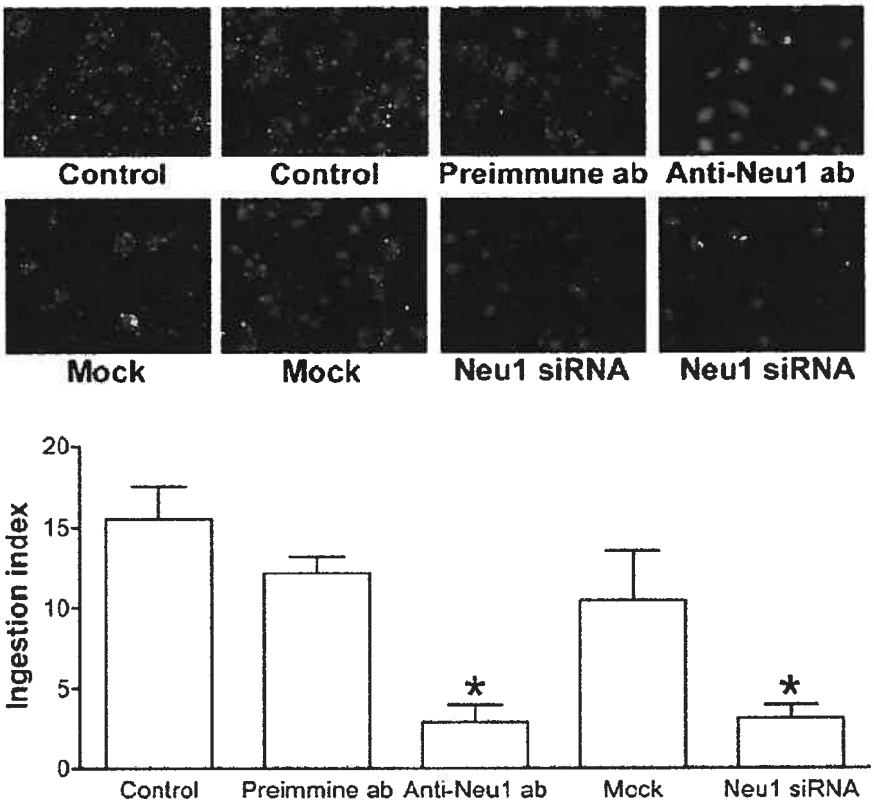

Control
Control
Preimmune ab
Anti-Neu1 ab
Mock
Mock
Neu1 siRNA
Neu1 siRNA
Ingestion index
20
15
10
5
0
*
*
Control
Preimmine ab
Anti-Neu1 ab
Mock
Neu1 siRNA

Figure 9

Figure 9. Reduced phagocytosis in THP-1-derived macrophages with suppressed Neu1.

Control THP-1 derived macrophages (***Control***), cells transfected with *Neu1* siRNA (***Neu1 siRNA***) and scrambled RNA (***Mock***) as well as the cells differentiated in the presence of 20 μg/ml of anti-Neu1 antibodies (***Anti-Neu1 ab***) or of the preimmune serum (***Preimmune ab***) were incubated for 3 h at 36°C in the presence of fluorescein-labeled *E. coli* bioparticles (30 particles per one cell), fixed, and stained with DAPI. Slides were examined on the Nikon Eclipse E6000 direct epifluorescence microscope. Magnification 200x. Panels represent typical images obtained in triplicate experiments; from 70 to 150 cells (10 panels) were studied for each condition in each experiment.

Lower panel. Ingestion index was calculated for each type of cells as the percentage of macrophages with phagocytized particles multiplied on the average number of particles phagocytized per ingested macrophage. The results show an average ±SD of 3 independent experiments. * $p<0.001$ as compared to nontransfected cells, mock-transfected cells or cells treated with preimmune serum.

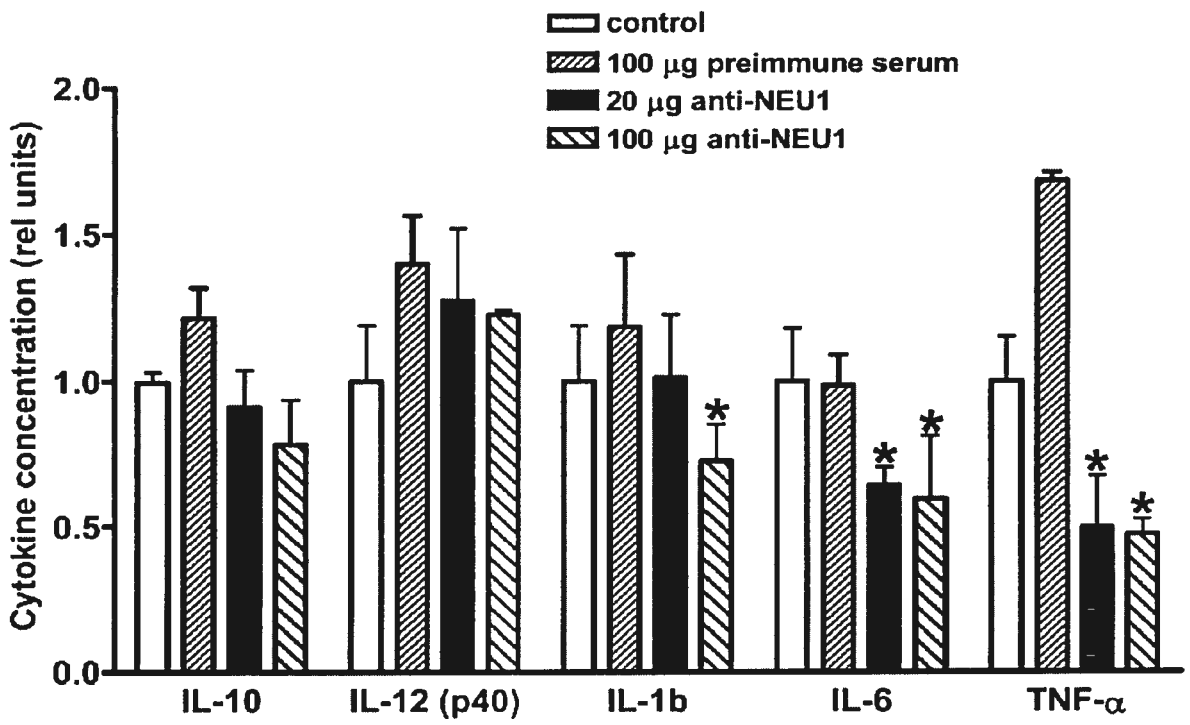
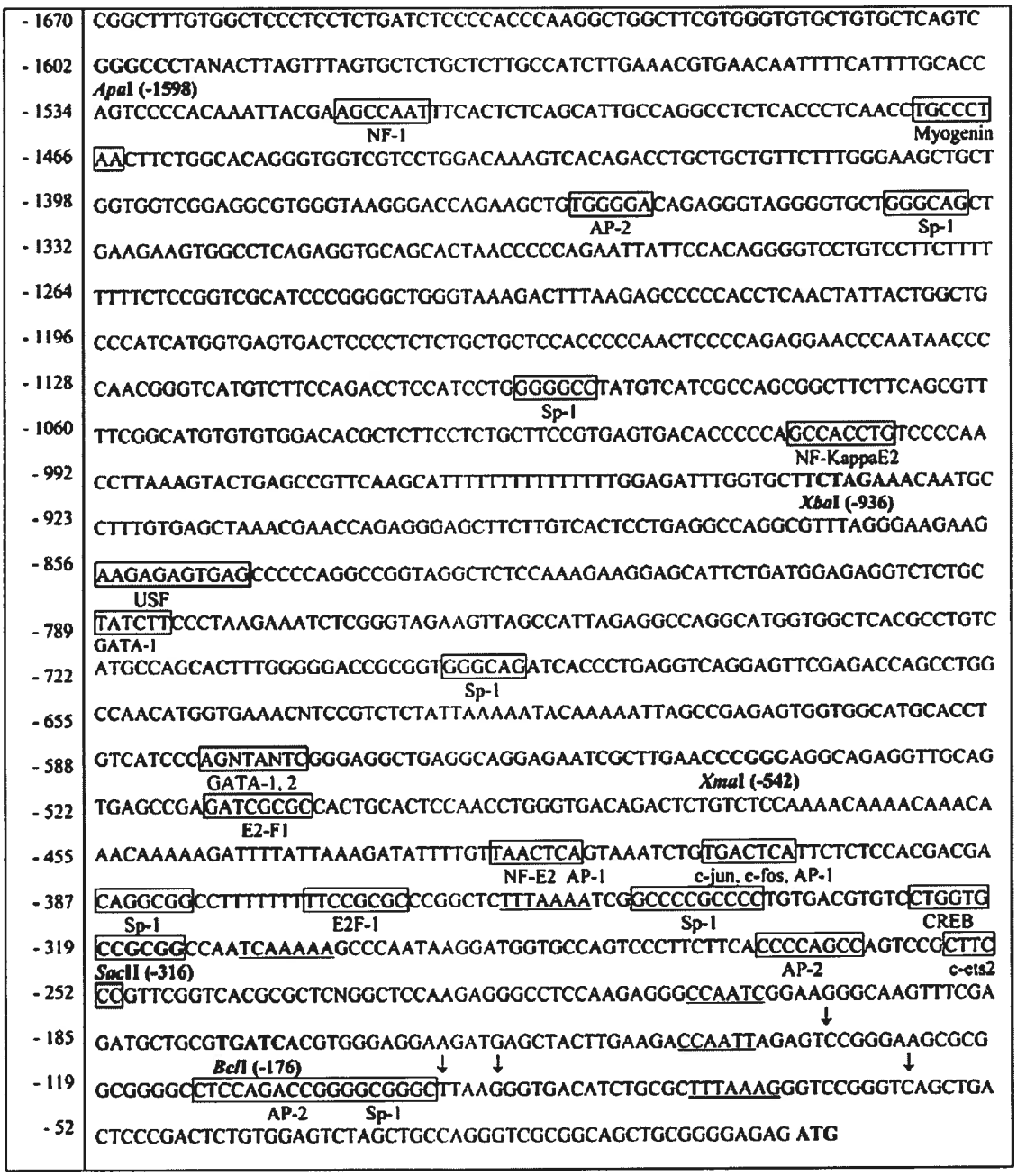

control
100 μg preimmune serum
20 μg anti-NEU1
100 μg anti-NEU1
Cytokine concentration (rel units)
2.0
1.5
1.0
0.5
0.0
IL-10
IL-12 (p40)
IL-1b
IL-6
TNF-α
*

Figure 10

Figure 10. Effect of anti-Neu1 antibodies on the ionomycin-induced production of IL-10, IL-12(p40), IL-1b, IL-6 and TNF-α in THP-1-derived macrophages. THP-1-derived macrophages were stimulated for 12 h with PMA (20 ng/ml) and ionomycin (1 μg/ml) in the absence (***Control***), or in the presence of preimmune rabbit serum (100 μg/ml) of anti-Neu1 serum (20 μg/ml and 100 μg/ml) followed by the analysis of IL and TNF-α production by enzyme-linked immunosorbent assay. The data show an average of 3 independent experiments ± standard error; * - $p<0.05$ using unpaired t test.



Supl. Figure 1

Supplemental Figure 1. Nucleotide sequence of the 5`-flanking region of the human *NEU1* gene.

Putative regulatory sequences predicted by the sequence analysis using TRANSFAC 4.0 software are boxed and their names are shown under the sequence. Positions of transcription initiation sites identified by Ribonuclease protection assay (C. Richard, unpublished) are marked by arrows. TATA and CAAT boxes are underlined. Endonuclease restriction sites used for the deletion mapping are shown in bold and their positions are indicated in parentheses.

Supplemental Table. PCR primers and conditions used to measure the concentration of Neu1 and Neu3 mRNA in THP-1 cells during PMA-induced differentiation.

Gene	Primers	PCR condition	Product length
Human Sialidase 1 (Neu1)	Forward: TCCAAGGCTGAGAACGACTT Reverse: AGGTGTCCACTGAGCCGAT	95°C 900 sec. 45 cycles of: 94°C 15 sec. 60°C 30 sec. 72°C 30 sec.	100 bp
Human Sialidase 3 (Neu3)	Forward: TCCACGAGGAGAGATGAGGA Reverse: GCTTCCATCAGTGGCTTCAG	95°C 900 sec. 45 cycles of: 94°C 15 sec. 60°C 30 sec. 72°C 30 sec.	100 bp
18S rRNA	Universal 18S Internal Standards primers (Ambion, Austin,TX)	95°C 900 sec. 35 cycles of: 94°C 15 sec. 63°C 30 sec. 72°C 15 sec.	315 bp

CHAPTER 4: NEU1 SIALIDASE BUT NOT CATHEPSIN A ACTIVITY ON THE SURFACE OF THE IMMUNE CELLS IS IMPORTANT FOR THE FUNCTIONAL INTEGRITY

ARTICLE 3

Manuscript, January, 2007

NEU1 SIALIDASE BUT NOT CATHEPSIN A ACTIVITY ON THE SURFACE OF THE IMMUNE CELLS IS IMPORTANT FOR THE FUNCTIONAL INTEGRITY

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ABSTRACT

The differentiation of monocytes as well the activation of lymphocytes and macrophages is accompanied by the appearance on their surface of 2 lysosomal enzymes, sialidase (neuraminidase 1, Neu1) and carboxypeptidase A (cathepsin A, CathA), which forms a complex with and activates Neu1. To clarify the biological roles of Neu1 and CathA in the immune cells we have developed animal models of a single CathA deficiency and a double CathA/Neu1 deficiency by gene targeting in mice. In the first line of mice, $CathA^{S190A}$, we have replaced the nucleophyl of the CathA active site, Ser190 with Ala, which abolished the enzymatic activity, while the mutant protein retained its ability to activate Neu1. The second line, $CathA^{S190A\text{-}Neo}$, was the hypomorphic CathA mice with the secondary ~90% reduction of the Neu1 activity. Macrophages derived from the splenocytes or the peripheral blood monocytes of the $CathA^{S190A\text{-}Neo}$ mice as well as their immature dendritic cells showed significantly reduced capacity to engulf bacteria. The mature dendritic cells also had lower effect on T cells proliferation, while properties of the cells derived from the $CathA^{S190A}$ mice were indistinct from those of the wild type controls. Macrophages from the Neu1-deficient mice showed an increased affinity to the sialic acid-binding lectin from *Maackia amurensis* consistent with a compromised processing of sialylated sugar residues of cell surface molecules. Altogether our data suggest that the cell surface Neu1 plays an important role in the regulation of affinity of the immune cells towards each other and external pathogens.

INTRODUCTION

Sialic acids are abundantly expressed on the surface of the immune cells and implicated in mediating recognition between the cells, between the cells and extracellular matrix as well as between the cells and a range of pathogenic viruses, bacteria and protozoa during the inflammatory and immune reactions (reviewed in Kelm and Schauer, 1997). For example, members of the siglecs (sialic acid binding IgG lectins) superfamily are known to mediate many of these interactions contributing, in particular, to scavenging function of macrophages, pathogen uptake and antigen presentation (reviewed in Munday et al., 1999). Much less is known about the role of sialidases and sialotransferases that can regulate cellular affinity by modifying the sialylation of cell surface molecules.

Previous data showed that the lysosomal sialidase encoded by the *NEU1* gene in addition to its role in intralysosomal catabolism may be also involved in cellular signalling during the immune response. In particular, during the activation of mouse T cells Neu1 is expressed on the plasma membrane and is required for the early production of IL-4 and for the interaction of T cells with the antigen presenting cells (Landolfi et al., 1986; Chen et al., 1997, 2000; Nan et al., 2006; Lukong et al., 2002). In addition, Neu1 of T cells converts the group specific component (Gc protein) into a factor necessary for the inflammation-primed activation of macrophages (Yamamoto et al., 1993, Naraparaju et al., 1994) T-cells derived from SM/J or B10.SM strains of mice, with the reduced Neu1 activity due to a missence mutation in the *Neu1* gene (Carrillo et al., 1997) fail to convert Gc and synthesize IL-4, while B cells of these mice cannot produce IgG_1 and IgE after the immunization with pertussis toxin (Landolfi et al., 1985, 1986; Naraparaju et al., 1994).

Previously we showed that Neu1 is increased up to 14-fold during the differentiation of human monocytes into macrophages (Stamatos et al., 2005). We further showed that the majority of Neu1 in macrophages is located on the cell surface (Liang et al., 2006). Activation and stabilization of the Neu1 in the lysosome, requires its association with the lysosomal multienzyme complex (LMC) which also containing the lysosomal carboxypeptidase A (cathepsin A, Cath A/protective

protein), ß-galactosidase and N-acetylgalactosamine-6-sulfate sulfatase (reviewed in Pshezhetsky and Ashmarina, 2001). Interestingly during the differentiation of macrophages, CathA but not other components of LMC is sorted to the plasma membrane similarly to Neu1. Both proteins were first targeted to the lysosome and then sorted to LAMP-2-negative, MHC II-positive vesicles, later merged with the plasma membrane (Liang et al., 2006).

In order to clarify the physiological role of both enzymes in the immune cells we have compared essential functional properties of monocyte-derived macrophages, dendritic cells (DCs) from the genetically targeted mice having either a single CathA or a double CathA/Neu1 deficiency. The obtained data suggest that the cell surface Neu1 regulates the affinity of the immune cells towards each other and external pathogens and is therefore necessary for their functional integrity.

EXPERIMENTAL PROCEDURES

Animals

The *CathA*^{S190A} mice carrying the point Ser190Ala mutation in the CathA gene and the *CathA*$^{S190A\text{-}Neo}$ mice carrying in addition a *PGK-neo* cassette in the intron 7 were developed through the targeted disruption of the CathA gene as described (Seyrantepe et al. submitted). The *CathA*$^{S190A\text{-}Neo}$ and *CathA*^{S190A} mice ranging from 4 to 12 weeks of age were compared with appropriate WT littermate controls. All mice were bred and maintained in the Canadian Council on Animal Care (CCAC)-accredited animal facilities of the Ste-Justine Hospital Research Center according to the CCAC guidelines. Approval for the animal care and the use in the experiments was granted by the Animal Care and Use Committee of the Ste-Justine Hospital Research Center.

Isolation and culturing of blood monocytes

Peripheral blood mononuclear cells (PBMCs) were isolated by leukophoresis of blood from mice heart followed by consequent centrifugations over Ficoll-Paque

Plus and Percoll (both Amersham Biosciences) gradients as described (Liang et al., 2006). To obtain monocyte-derived macrophages, the cells were suspended at 2 x 10^6 cells/ml in RPMI medium 1640 (Gibco, Grand Island, NY) containing 10% heat-inactivated fetal calf serum (FCS). After 2 hrs, non-adherent cells were removed by two washes with phosphate-buffered saline (PBS) and the adherent monocytes were maintained at 4 x 10^6 cells/well in 6 well tissue culture plates (Costar, Corning Inc., Corning, NY) at 37°C in a humidified atmosphere containing 5% CO_2. After 7 days differentiated macrophages (larger and more granular than monocytes as seen by light microscopy) were harvested in PBS by gentle scraping with a polyethylene cell scraper (Nalge Nunc International, Rochester NY). The harvested cells were confirmed to have characteristic macrophage cell surface phenotypic markers ($CD14^+$, $CD206^+$) by flow cytometry.

Isolation and culturing of splenocytes

Fresh splenocytes were obtained by teasing the mouse spleens under aseptic conditions. Erythrocytes were removed by centrifugation over Ficoll-Paque Plus gradient and the remaining unfractionated nucleated spleen cells were washed twice with PBS and adjusted to a density of 7.5 x 10^5 of cells per ml of RPMI medium 1640 containing 10% of heat-inactivated FCS. To obtain splenocyte-derived macrophages, purified splenocytes were maintained at 2 x 10^6 of cells per well in 6 well tissue culture plates (Costar, Corning Inc., Corning, NY) at 37°C in a humidified atmosphere containing 5% CO_2. At the specified times, non-adherent cells were removed by two washes with PBS and the adherent, differentiated macrophages were harvested in PBS by gentle scraping with a polyethylene cell scraper (Nalge Nunc International, Rochester, NY). The harvested cells were confirmed to have characteristic macrophage cell surface phenotypic markers (CD14+, CD206+) by flow cytometry.

Immature splenocyte-derived DCs were obtained as described earlier (Berthier et al., 2000). Briefly, purified mouse splenocytes were suspended at a density of 7.5 x 10^5 of cells per ml in Iscove's modified Dulbecco's medium (IMDM)

(Gibco, Life Technologies, Grand Island, NY) supplemented with 12.5% heat inactivated FCS, non essential amino acids (0.1 mM/ml), sodium pyruvate (1 mM/ml), 2-mercaptoethanol (50 μM), penicillin (5000 U/ml), streptomycin (50 mg/ml) and fungizone (0.5%). Two ml of this suspension were plated per 35 mm tissue culture dish (Nunclon, Glostrup, Denmark) and culture development proceeded over two different steps: the first step was devised to favour the proliferation of DC progenitors by early cytokine IL-6 (R&D Systems, Abingdon, UK) and the second, to induce the maturation of DC with cytokines flt-3L and GM-CSF (R&D Systems) known to exert a differentiation activity on mouse DC. The early phase lasted from day 0 to day 6 of incubation when the proliferation of DC progenitors took place under the influence of IL-6 (25 ng/ml). The second culture phase started on day 6 when IL-6 was withdrawn from the medium. The cells were washed, counted, split to a concentration of 1.5 x 10^5 of cells per ml in fresh complete medium and cultured in the presence of flt-3L (25 ng/ml) and GM-CSF (1000 U/ml) during the next 3 to 4 weeks. The cells were split again on days 10, 14, 20 and 26 by replating at 4 to 6 x 10^5 of cells per ml. To obtain mature splenocyte-derived DCs, the cells were cultured for an additional 48 hrs in the presence of 10 μg/ml of lipopolysaccharides (LPS, *E. coli* 026:B6, Sigma, St. Louis, MO, USA).

Enzyme assays

Sialidase activity in cellular homogenates was assayed using the synthetic fluorogenic substrate 2`-(4-methylumbelliferyl)−α−D-N-acetylneuraminic acid (4MU-NeuAc; Sigma) as described (Potier et al., 1979) with the following modifications. Freshly isolated cells (2-5 x 10^6) were homogenized in 0.20 ml of a solution containing 0.5% (v/v) Triton X-100, 0.05 M sodium acetate, pH 4.4 by sonication and incubated with 0.125 mM 4MU-NeuAc at 37°C for 1 h. The reaction was terminated by the addition of 1.9 ml of a solution containing 0.15 M glycine, pH 10.5. Liberated 4-methylumbelliferone was measured using a Shimadzu RF-5301 spectrofluorometer with excitation at 355 nm and emission at 460 nm. Enzyme activity is expressed as the conversion of 1 nmol of substrate per hr. Protein

concentration was measured by the Bradford method using a protein assay kit (Bio-Rad, Hercules, CA, USA) and the amount of activity measured in each sample was corrected based on protein concentration to represent activity per milligram of protein.

β-Hexosaminidase activity in cellular homogenates was assayed with 1.25 mM 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside as previously described (Rome et al., 1979). After the incubation at 37°C for 15 min, the reaction was terminated with 1.9 ml of 0.4 M glycine buffer, pH 10.4, and the fluorescence of the liberated 4-methylumbelliferone was measured as described above. The carboxypeptidase activity of cathepsin A was measured as follows. Freshly isolated cells (2-5 x 10^6) were homogenized in 0.20 ml of a water containing 0.5% (v / v) Triton X-100 and incubated with 100 µl of 1.5 mM CBZ-Phe-Leu (Sigma-Aldrich, St. Louis, MO, USA) in 100 mM sodium acetate buffer, pH 5.2 at 37°C for 30 min. The reaction was terminated by addition of 20 µl of trichloroacetic acid. Precipitate was removed by 10 min centrifugation at 12,000g and 100 µl of the supernatant was mixed with 3 ml of the 0.05 M sodium borate buffer pH 9.5 containing phthaldialdehyde (Sigma, 0.15 mg/ml) and β-mercaptoethanol (0.0075%). After 5 min incubation the concentration of leucine was measured using a Shimadzu RF-5301 spectrofluorometer with excitation at 340 nm and emission at 455 nm (Roth, 1973).

Phagocytosis assay

The splenocyte-derived macrophages or immature DCs seeded on cover slips were incubated for 3 hours with fluorescein-labeled *E. coli* (K-12 strain) bioparticles (Molecular Probe, Eugene, OR) in a ratio of 50 particles per one cell at 37°C or 4°C, washed 3 times with cold PBS, fixed with 4% paraformaldehyde for 1 h, permeabilized with 0.5% Triton X-100, and washed twice with 0.05% Tween-20 in PBS. Cells were counterstained with 3 µM DAPI and washed again with 0.05% Tween-20 in PBS. Slides were examined using the Nikon Eclipse E6000

epifluorescence microscope and internalization was quantitated by flow cytometry using FACS Calibur.

Immunofluorescent labeling and flow cytometry

Monoclonal antibodies, conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE), against MHC class II (anti-I-A), HL3–PE (anti-CD11c), RMMP-1 (anti-CD86–PE, B7-2), CD45 and CD14 were purchased from eBioscience (San Diego, CA). For surface immunostaining adherent cells were detached by pipeting after 3-5 minutes incubation at 37°C in PBS containing 3mM EDTA. Aliquots of 1 x 10^6 cells were incubated with 500 ng/ml of mouse IgG1 for 10 minutes on ice in order to block Fc receptors, followed by a double staining with the appropriate antibodies for 30 minutes on ice and in the dark. Cells were washed three times with PBS containing 0.05% (w/v) BSA and fixed with 2% paraformaldehyde. The acquisition was done using a FACS Calibur (Beckton Dickinson, San Diego, USA) and data were analysed using the CellQuest Pro software.

Lymphocyte proliferation assay

Mice lymphocytes were isolated by collecting the non-adherent cells from splenocytes of normal mice. Purified mice lymphocytes were maintained at 37°C in a humidified atmosphere of 5% CO_2 in RPMI 1640 medium, containing 5,000 units of Penicillin/Streptomycin and Fungizone (both Life Technologies, Inc.). Mice lymphocytes (2 x 10^6 in 3 ml medium) were seeded in six-well tissue culture plates and incubated at 37°C in 5% CO_2 in the presence or absence of mature splenocyte-derived DC (1 x 10^5). Cells were exposed to 1 μCi/ml of [methyl-^{3}H]-thymidine (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA). Ninety-six hours after the exposure to [methyl-^{3}H]-thymidine the cells were first washed twice with PBS and then four times with 5% trichloroacetic acid (ACP Chemicals) in water. The cell pellets were solubilized in 200 μl of 0.2% Triton X-100, 0.1 M NaOH. The radioactivity in the cell homogenates was measured using a Top Count instrument (Packard). At 24, 48 and 96 hrs cells were counted using haemacytometer (Fisher).

Lectin affinity assay

The splenocyte-derived macrophages seeded on cover slips were incubated for 15 min at 37°C with 100 μg/ml of fluorescein-labeled *Maackia amurensis* lectin (EY Laboratories, INC, San Mateo, CA). Then the cells were washed 3 times with cold PBS and fixed with 4% paraformaldehyde for 1 h. Cells were counterstained with 3 μM DAPI and washed again with 0.05% Tween-20 in PBS. Slides were examined using the Nikon Eclipse E6000 epifluorescence microscope.

RESULTS

Immune cells derived from the *CathA*$^{S190A\text{-}Neo}$ but not from the *CathA*^{S190A} mice show the reduced sialidase activity

Our previous studies performed on the tissues from kidney, livers, brain and lungs of the *CathA*$^{S190A\text{-}Neo}$ and the *CathA*^{S190A} mice (Seyrantepe et al., submitted) showed that both mice had almost zero level of CathA activity, consistent with the presence of the Ser190Ala point mutation which eliminates the active site nucleophile (Galjart et al., 1991). In contrast, a significant difference was observed in the tissue levels of the sialidase activity measured at acidic pH 4.5 with the synthetic fluorogenic substrate 2`-(4-methylumbelliferyl)−α−D-N-acetylneuraminic acid (4MU-NeuAc). *CathA*^{S190A} mice showed normal activity levels, whereas in tissues of the *CathA*$^{S190A\text{-}Neo}$ mice, that has a *PGK-neo* cassette inserted into the intron 7 of the *CathA* gene the sialidase activity was reduced to ~10%. Further studies showed that the *CathA*^{S190A} mice express normal levels of inactive CathA protein capable of forming a complex with and activating the Neu1 sialidase. In the tissues of the *CathA*$^{S190A\text{-}Neo}$ mice the *CathA* mRNA and protein levels were dramatically reduced consistent with previously reported hypomorphic effect of the *neo* gene and caused the secondary deficiency of the Neu1 (Seyrantepe et al., submitted). Both the *CathA*$^{S190A\text{-}Neo}$ and *CathA*^{S190A} mice are vital, fertile, grow normally and have a normal lifespan. The FACS calibre immunophenotypic profiling of total splenocytes obtained from the WT, *CathA*^{S190A} and *CathA*$^{S190A\text{-}neo}$ mice and double-stained with anti-MHC

and anti-CD-14 monoclonal antibodies did not reveal any difference in the splenal population of monocytes and immature DCs.

In order to find whether the immune cells of the *CathA*$^{S190A\text{-}Neo}$ and the *CathA*^{S190A} mice show decrease in the CathA and sialidase activities similar to that observed in the non-lymphatic tissues we have assayed both activities in the blood monocytes and in the monocyte-derived macrophages. We have also assayed CathA and sialidase activities in the monocytes purified from the spleen tissues, as well as in splenocytes-derived macrophages, immature and mature DCs. As we expected, only background levels (10-15%) of the carboxypeptidase activity measured with the specific CathA substrate CBZ-PheLeu could be detected in blood monocytes and macrophages from both the *CathA*$^{S190A\text{-}Neo}$ and the *CathA*^{S190A} mice (Fig 2A). This activity does not change upon the differentiation and reflects the background level of CBZ-Phe-Leu conversion by other cellular carboxypeptidases. In the cells from the WT mice the CathA activity increases but not significantly (Fig. 2A). Similar results were obtained also for the spleen-derived macrophages (Fig. 2A) and DCs (not shown). In contrast, a significant increase of the sialidase activity was observed during the differentiation of the monocytes derived from the blood or the spleens of the wild-type and *CathA*^{S190A} mice (Fig. 2B). As compared to both spleen and blood-derived monocytes the sialidase activity was increased at least 2 fold in macrophages, at least 4 fold in immature DCs, and at least 5-fold in the mature DCs. Macrophages from the *CathA*$^{S190A\text{-}Neo}$ mice (both blood- and spleen-derived) showed sialidase activity almost similar to that in the corresponding monocytes, confirming our previous finding that the increased sialidase activity in macrophages results from the induced expression of the *Neu1* gene. Although the sialidase activity in immature and mature DCs from the *CathA*$^{S190A\text{-}Neo}$ mice is increased it still remains significantly lower then in the cells from the WT or the *CathA*^{S190A} mice. The control lysosomal enzyme, β-hexosaminidase showed the same activity in the cells from all mice (not shown).

Macrophages and immature DCs from the *CathA*$^{S190A\text{-}Neo}$ mice show impaired capacity to internalize pathogens

In order to understand whether the Neu1 and/or CathA activities are important for the primary function of macrophages and immature DCs we have studied the phagocytic capacity of these cells derived from the wild-type, *CathA*$^{S190A\text{-}Neo}$ and *CathA*^{S190A} mice. The macrophages derived from the splenocytes of the WT, CathA$^{S190A\text{-}Neo}$ and *CathA*^{S190A} mice were incubated for 3 hours at 37° or 4°C with the fluorescein-labeled *E. coli* bioparticles, washed, fixed with paraformaldehyde, stained with DAPI and studied using an epifluorescent microscope. The ingestion index of macrophages was calculated as a percentage of macrophages with engulfed particles multiplied by the average number of particles phagocytized per ingested macrophage. The control level measured at 4°C was subtracted from all values. No statistical difference was observed between the ingestion index of macrophages derived from the *CathA*^{S190A} (21.5±4.9) and the WT (19.3±5.3) mice (Figure 3 A). In contrast, the ingestion index for the macrophages derived from the *CathA*$^{S190A\text{-}Neo}$ mice (9.2±2.0) was statistically reduced as compared to the cells from both the WT and the *CathA*^{S190A} (Figure 3 A). Similar results were also obtained for the macrophages (peritoneal adhesive cells) purified from the peritoneal cavities. The cells derived from the *CathA*$^{S190A\text{-}Neo}$ showed ingestion index at least twice lower than that of the cells from the WT and *CathA*^{S190A} mice.

Significant difference between the phagocytic activity of the macrophages from the WT and *CathA*$^{S190A\text{-}Neo}$ mice was also observed by flow cytometry (Figure 3 B). After 3hr incubation with fluorescein-labeled *E. coli* bioparticles, more than 44% of the splenocyte-derived macrophages from the WT mice were positive for fluorescein, whereas only 18% of fluorescein positive cells were observed among the cells from the *CathA*$^{S190A\text{-}Neo}$ mice (Figure 3 B).

As compared to the macrophages, the spenocyte-derived immature DCs have significantly enhanced phagocytic activity. When immature DCs derived from the splenocytes of the WT or the *CathA*^{S190A} mice were incubated for 3 hours with the fluorescein-labeled *E. coli* bioparticles their ingestion index was ranging between 35

and 42. The ingestion index of the immature DCs from the *CathA*$^{S190A\text{-}Neo}$ mice was reduced to 18.8±3.2 (Figure 3 C).

Mature DCs from the *CathA*$^{S190A\text{-}Neo}$ mice show reduced capacity to activate lymphocyte proliferation

The ability to activate proliferation of lymphocytes is one of the essential physiological properties of the mature DCs. To understand if this function depends on the Neu1 expression we have obtained populations of the mature DCs from the WT, the *CathA*^{S190A} and the *CathA*$^{S190A\text{-}Neo}$ mice by 48-hr treatment of the splenocytes-derived immature DCs with LPS and studied their ability to induce the proliferation of lymphocytes. Mouse lymphocytes were isolated by collecting the non-adherent cells from splenocyte cultures of the WT siblings, the *CathA*^{S190A} and the *CathA*$^{S190A\text{-}Neo}$ mice and placed in aliquots of 2 x 10^6 cells in 3 ml medium into the culture plates containing 1 x 10^5 of mature splenocyte-derived DCs in the presence of [methyl-^{3}H]-thymidine (1 μCi/ml). After 96 hrs of incubation cells were harvested and the total radioactivity was measured in the cell homogenates (Figure 4 B). At 24, 48 and 96 hrs the cells were counted using haemocytometer (Figure 4 A). No proliferation of lymphocytes was observed in the absence of DCs. The DCs from both the WT and the *CathA*$^{S190A\text{-}Neo}$ mice stimulated cell proliferation but ~20% slower growth rate was observed in the presence of the cells from the *CathA*$^{S190A\text{-}Neo}$ mice as compared to the WT controls. Similarly, ~25% lower transcriptional activity of lymphocytes (measured as the amount of [methyl-^{3}H]-thymidine incorporated by the cells within 96 hrs) was observed in the presence of the mature DCs from the *CathA*$^{S190A\text{-}Neo}$ mice then in the presence of the mature DCs from the WT mice (Figure 4 B).

Macrophages derived from the Neu1-deficient mice show higher affinity towards the sialic acid binding lectin as compared with the cells from the WT or CathA-deficient mice.

In order to understand whether Neu1 is involved in macrophages in processing the sialylated macromolecules present on the cell surface we have studied

the affinity of the cells derived from the wild-type, *CathA*$^{S190A\text{-}Neo}$ and *CathA*^{S190A} mice towards the sialic acid-binding lectin from *Maackia amurensis*. The macrophages were incubated with the fluorescein-labelled lectin, washed, fixed with paraformaldehyde, stained with DAPI and studied using an epifluorescent microscope. We found that the macrophages from the *CathA*$^{S190A\text{-}Neo}$ mice showed intensive peripheral staining with the lectin (Figure 5) consistent with the high silyalation of their surface. The cells from the CathA-deficient *CathA*^{S190A} mice showed weak staining similar to that of the cells from the WT mice.

DISCUSSION

Growing body of evidence suggests that in addition to their role in catabolism of macromolecules lysosomal enzymes have important physiological functions outside the lysosomes, most often on the cell surface or outside the cell. In particular, the lysosomal β–hexosaminidase A has been recently shown to participate in the production of ceramide from GM3 ganglioside on the external leaflet of plasma membrane of fibroblasts (Mencarelli et al., 2005; Valaperta et al., 2006). In similar fashion, the components of the multienzyme lysosomal complex, Neu1, CathA and β-galactosidase (in its alternatively spliced form) have important physiological functions outside the lysosome participating in the processing of endothelin-1 (ET-1), a potent vasoconstrictive peptide, which plays multiple roles in nonvascular tissues (Itoh et al., 1995), assembly of the elastic fibres (Hinek et al., 1993; 2006) and cell signalling during the immune response (Stamatos et al., 2005; Liang et al., 2006). In humans inherited mutations in CathA cause disruption of the complex and trigger the galactosialidosis (OMIM 256540), an autosomal recessive disease characterized by combined deficiency of Neu1, ß-galactosidase and CathA (reviewed in Thomas et al., 2001). Another autosomal recessive disease, sialidosis (OMIM 256550), is caused by mutations directly affecting the *NEU1* gene (reviewed in d'Azzo et al. 2001; Pshezhetsky and Ashmarina, 2001). Several phenotypic characteristics of patients, including abnormal, arterial hypertension and cardiomyopathies related to defects in elastic fiber formation, are not predicted from the lysosomal hydrolase functions of these proteins, but consistent with the disruption of their secondary activities on the cells surface, related to hemodynamic functions and formation of cartilage (Caciotti et al., 2005; Kyllerman et al., 1993). However no data exist clarify whether the Neu1 and CathA deficiencies affect immune responses in the galactosialidosis and sialidosis patients.

Recently we have developed animal models of single CathA deficiency and double CathAA/Neu1 deficiency by gene targeting in mice (Seyrantepe et al., submitted). In the first line of mice ($CathA^{S190A}$) we have replaced the nucleophyl of the CathA active site, Ser190 with Ala, which abolished the enzymatic activity, while

the mutant protein retained its ability to activate Neu1. We have also produced a line (*CathA*$^{S190A\text{-}Neo}$ mice) that had a *PGK-neo* cassette inserted in the *CathA* gene. These mice had a significantly reduced (3-10%) Neu1 activity, due to dramatically decreased *CathA* mRNA levels, consistent with previously reported hypomorphic effects of the *neo* gene (Meyers et al., 1998; Carmeliet et al., 1996; Moran et al., 1999). Therefore, we have obtained a hypomorphic CathA mice with the secondary deficiency of Neu1 activity. Since the residual level of the Neu1 activity in the tissues of the *CathA*$^{S190A\text{-}Neo}$ mice is still higher than that in previously described knockout CathA mice (Zhou et al., 1995), the animals do not develop severe galactosialidosis phenotype.

Our data strongly indicate that the immune cells from the Neu1/CathA-deficient *CathA*$^{S190A\text{-}Neo}$ mice but not from the CathA-deficient *CathA*^{S190A} mice have a significantly reduced functional capacity. First we found that the spenocyte- and PBMC-derived macrophages, peritoneal adherent cells as well as the splenocytes-derived immature DCs were compromised in their ability to phagocytose pathogens (Figure 3). This in turn could mean that these cells are less efficiently activated, which is consistent with our previous observation that the THP-1-derived macrophages showed reduced production of cytokines including IL-1b, IL-6 and TNF-α after the Neu1 on the their surface was inhibited with the anti-Neu1 antibodies (Liang et al., 2006). In addition, the macrophages from the Neu1-deficient mice showed higher affinity to the FITC conjugated *Maackia amurensis* lectin (Figure 5) suggesting that they have increased sialylation of the cell surface glycoproteins and that Neu1 plays a major role in processing of the sialylated molecules on the cell surface.

Previously, optimal sialylation of both siglecs and their targets has been shown to be important for the cell-cell and cell-pathogen interactions of macrophages, DC, T, B and NK cells (reviewed in Crocker et al, 2002), therefore the latter could be affected by compromised processing of the sialoglycoconjugates in the *CathA*$^{S190A\text{-}Neo}$ mice. Indeed we have observed a significantly the reduced potency of the mature DCs in the activation of T-cell proliferation (Figure 4). The hosts

defective in these parameters may be predisposed to repeated infections with bacterial and viral pathogens, a condition described in the patients suffering from the lysosomal storage diseases (G. Mitchell, personal communication).

In conclusion, our data show that the cell surface Neu1 plays an important role in the regulation of affinity of the immune cells towards each other and external pathogens. Although further studies are required to understand how the partial Neu1 deficiency in the *CathA*$^{S190A\text{-}Neo}$ mice affect their immune response *in vivo*, our findings suggest that similarly to CD63, CD152, CD206 and other proteins with lysosomal/endosomal proteins expressed on the surface of the immune cells, Neu1 is important for their functional integrity.

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REFERENCES

Kelm, S., Schauer, R. (1997) *Int. Rev. Cytol.* 175, 137–240.

Munday J, Floyd H, Crocker PR. (1999) Sialic acid binding receptors (siglecs) expressed by macrophages. *J Leukoc Biol.* 66(5): 705-711.

Chen XP, Ding X, Daynes RA. Ganglioside control over IL-4 priming and cytokine production in activated T cells. *Cytokine* 12(7):972-985 (2000).

Chen XP, Enioutina EY, Daynes RA. The control of IL-4 gene expression in activated murine T lymphocytes: a novel role for neu-1 sialidase. *J Immunol* 158(7):3070-3080 (1997).

Landolfi NF, Cook RG. Activated T lymphocytes express class I molecules which are hyposialylated compared to other lymphocyte populations. *Mol Immunol* 23(3):297-309 (1986).

Nan X, Carubelli I, Stamatos NM. Sialidase expression in activated human T lymphocytes influences production of IFN-γ *J Leukoc Biol.*

Yamamoto N, Kumashiro R. Conversion of vitamin D3-binding protein (group-specific component) to a macrophage activating factor by the stepwise action of β-galactosidase of B cells and sialidase of T cells. *J Immunol* 151(5):2794-2802 (1993).

Naraparaju VR, Yamamoto N. Roles of β-galactosidase of B lymphocytes and sialidase of T lymphocytes in inflammation-primed activation of macrophages. *Immunol Lett* 43(3):143-148 (1994).

Carrillo MB, Milner CN, Ball ST, Snock M, Campbell RD. Cloning and characterization of sialidase from the m urine histocompatibility-2 complex: low levels of mRNA and a single amino acid mutation are responsible for reduced sialidase activity in mice carrying the Neu1a allele. *Glycobiology* 7:975-986 (1997).

Landolfi NF, Leone J, Womack JE, Cook RG. Activation of T lymphocytes results in an increase in H-2-encoded neuraminidase. *Immunogenetics* 22(2):159-167 (1985).

Lukong KE, Seyrantepe V, Landry K, Trudel S, Ahmad A, Gahl WA, Lefrancois S, Morales CR, Pshezhetsky AV. Intracellular distribution of lysosomal sialidase is controlled by the internalization signal in its cytoplasmic tail. *J Biol Chem.* (2001) 276(49): 46172-46181.

Pshezhetsky, A. V. and Ashmarina, M. (2001) *Prog.Nucleic Acid Res.Mol.Biol.* 69, 81-114

Potier M, Mameli L, Belisle M, Dallaire L, Melancon SB. Fluorometric assay of neuraminidase with a sodium (4-methylumbelliferyl-α-D-N-acetylneuraminate) substrate. *Anal Biochem.* (1979) 94(2):287-296.

Mencarelli S, Cavalieri C, Magini A, Tancini B, Basso L, Lemansky P, Hasilik A, Li YT, Chigorno V, Orlacchio A, Emiliani C, Sonnino S. Identification of plasma membrane associated mature beta-hexosaminidase A, active towards GM2 ganglioside, in human fibroblasts. *FEBS Lett.* 2005 Oct 24;579(25):5501-6.

Valaperta R, Chigorno V, Basso L, Prinetti A, Bresciani R, Preti A, Miyagi T, Sonnino S. Plasma membrane production of ceramide from ganglioside GM3 in human fibroblasts. *FASEB J.* 2006 Jun;20(8):1227-9.

N.J. Galjart, H. Morreau, R. Willemsen, N. Gillemans, E.J. Bonten, and A. d'Azzo, Human lysosomal protective protein has cathepsin A-like activity distinct from its protection function. *J. Biol. Chem.* 266, 14754-14762 (1991).

Hinek A, Rabinovitch M, Keeley F, Okamura-Oho Y, Callahan J. The 67-kD elastin/laminin-binding protein is related to an enzymatically inactive, alternatively spliced form of β-galactosidase. *J Clin Invest* 91(3):1198-1205 (1993).

Liang F, Seyrantepe V, Landry K, Ahmad R, Ahmad A, Stamatos NM, Pshezhetsky AV. Monocyte differentiation up-regulates the expression of the lysosomal sialidase, Neu1, and triggers its targeting to the plasma membrane via major histocompatibility complex class II-positive compartments. *J Biol Chem.* (2006) 281(37):27526-27538

Stamatos NM, Liang F, Nan X, Landry K, Cross AS, Wang LX, Pshezhetsky AV. Differential expression of endogenous sialidases of human monocytes during cellular differentiation into macrophages. *FEBS J.* (2005) 272(10):2545-2556.

Hinek A, Pshezhetsky AV, von Itzstein M, Starcher B. Lysosomal sialidase (neuraminidase-1) is targeted to the cell surface in a multiprotein complex that facilitates elastic fiber assembly. *J Biol Chem.* (2006) 281(6):3698-3710.

Thomas GH. Disorders of glycoprotein degradation: α-mannosidosis, β-mannosidosis, fucosidosis, and sialidosis. In: Scriver CR, Beaudet AL, Sly WS, Valle D, editors. The Metabolic and Molecular Bases of Inherited Disease. New York: McGraw-Hill. p 3507-3534 (2001).

Itoh K, Kase R, Shimmoto M, Satake H, Suzuki Y. Protective protein as an endogenous endothelin degradation enzyme in human tissues. *J Biol Chem* 270:515-518 (1995).

d'Azzo, A., Andria, G., Strisciuglio, G., and Galjaard, H. (2001) Galactosialidosis. In Scriver, C. R., Beaudet, A. L., Sly, W. S., and Valle, D., editors. *Metabolic and Molecular Bases of Inherited Disease*, McGraw-Hill, New York

Pshezhetsky, A. V. and Ashmarina, M. (2001) *Prog.Nucleic Acid Res.Mol.Biol.* 69, 81-114 Caciotti A, Donati MA, Bardelli T, d'Azzo A, Massai G, Luciani L, Zammarchi E, Morrone A. Primary and secondary elastin-binding protein defect leads to impaired elastogenesis in fibroblasts from G_{M1}-gangliosidosis patients. *Am J Pathol.* (2005) 167(6):1689-98.

Caciotti A, Donati MA, Boneh A, d'Azzo A, Federico A, Parini R, Antuzzi D, Bardelli T, Nosi D, Kimonis V, Zammarchi E, Morrone A. Role of β-galactosidase and elastin binding protein in lysosomal and nonlysosomal complexes of patients with G_{M1}-gangliosidosis. *Hum Mutat.* (2005) 25(3):285-92.

Kyllerman M, Mansson JE, Westphal O, Conradi N, Nellstrom H. Infantile galactosialidosis presenting with congenital adrenal hyperplasia and renal hypertension. *Pediatr Neurol* 9(4): 318-322 (1993).

Meyers EN, Lewandoski M, Martin GR. An Fgf8 mutant allelic series generated by Cre- and Flp-mediated recombination. *Nat Genet* 18(2):136-141 (1998).

Carmeliet P, Ferreira V, Breier G, et al. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* 380(6573):435-439 (1996).

Moran JL, Levorse JM, Vogt TF. Limbs move beyond the radical fringe. *Nature* 399(6738):742-743 (1999).

Zhou XY, Morreau H, Rottier R, Davis D, Bonten E, Gillemans N, Wenger D, Grosveld FG, Doherty P, Suzuki K, Grosveld G, d'Azzo A. Mouse model for the lysosomal disorder galactosialidosis and correction of the phenotype with overexpressing erythroid precursor cells. *Genes Dev* 9:2623-2634 (1995).

Crocker, P. R. (2002) *Curr.Opin.Struct.Biol.* 12, 609-615

Berthier R, Martinon-Ego C, Laharie AM and. Marche PN. A two-step culture method starting with early growth factors permits enhanced production of functional dendritic cells from murine splenocytes. *J Immunol Methods.* May 26;239(1-2):95-107. (2000)

Roth M, Fluorescence reaction for amino acids. *Anal Chem.* Jun;43(7):880-2 (1971)

Rome LH, Garvin AJ, Allietta MM & Neufeld EF. Two species of lysosomal organelles in cultured human fibroblasts. *Cell* 17, 143–153.(1979)

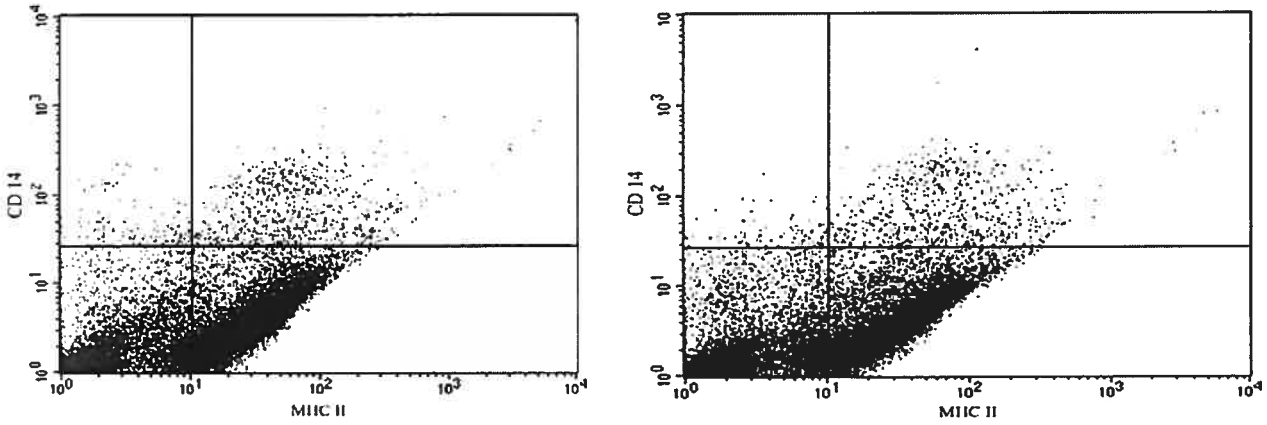
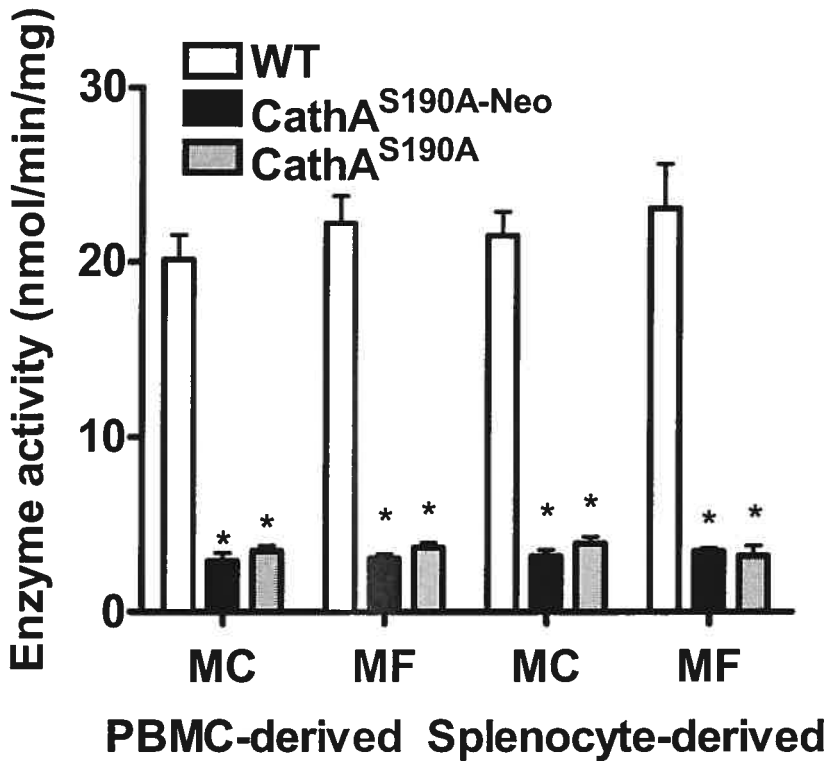


Figure 1

Figure 1. FACS calibre immunophenotypic profiles of total splenocytes obtained from the WT, and *CathA*$^{S190A\text{-}neo}$ mice.

The cells were double-stained with FITC-conjugated anti-MHC monoclonal antibodies, II-A–FITC and PE-conjugated anti-CD-14 monoclonal antibodies, CD14-PE. The results are representative of three separate experiments.

A


WT
CathA S190A-Neo
CathA S190A
Enzyme activity (nmol/min/mg)
30
20
10
0
MC
MF
MC
MF
PBMC-derived
Splenocyte-derived

B

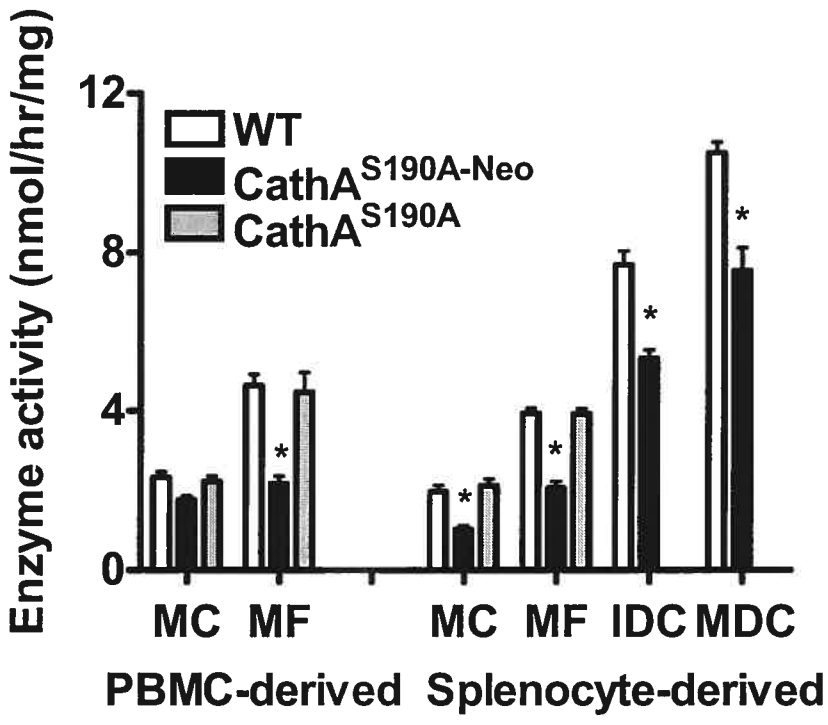

WT
CathA S190A-Neo
CathA S190A
Enzyme activity (nmol/hr/mg)
12
8
4
0
MC
MF
MC
MF
IDC
MDC
PBMC-derived
Splenocyte-derived

Figure 2

Figure 2. **A.** Specific CathA activity measured with the peptide substrate CBZ-PheLeu at pH 5.2 in total homogenates of blood-derived and spleen-derived monocytes (**MC**) and macrophages (**MF**). Values represent means ± S.D. of 3 independent experiments. * $p < 0.05$

B. Specific activity of sialidase against the synthetic substrate 4MU-NeuAc at pH 4.5 was measured in total homogenates of blood-derived and spleen-derived monocytes (**MC**) and macrophages (**MF**) as well as in the splenocyte-derived immature (**IDC**) and mature (**MDC**) dendritic cells. Splenocyte-derived mature dendritic cells were induced by 10 μg/ml of lipopolysaccharides for 48 hours. Values represent means ± S.D. of triplicate experiments. * $p < 0.05$

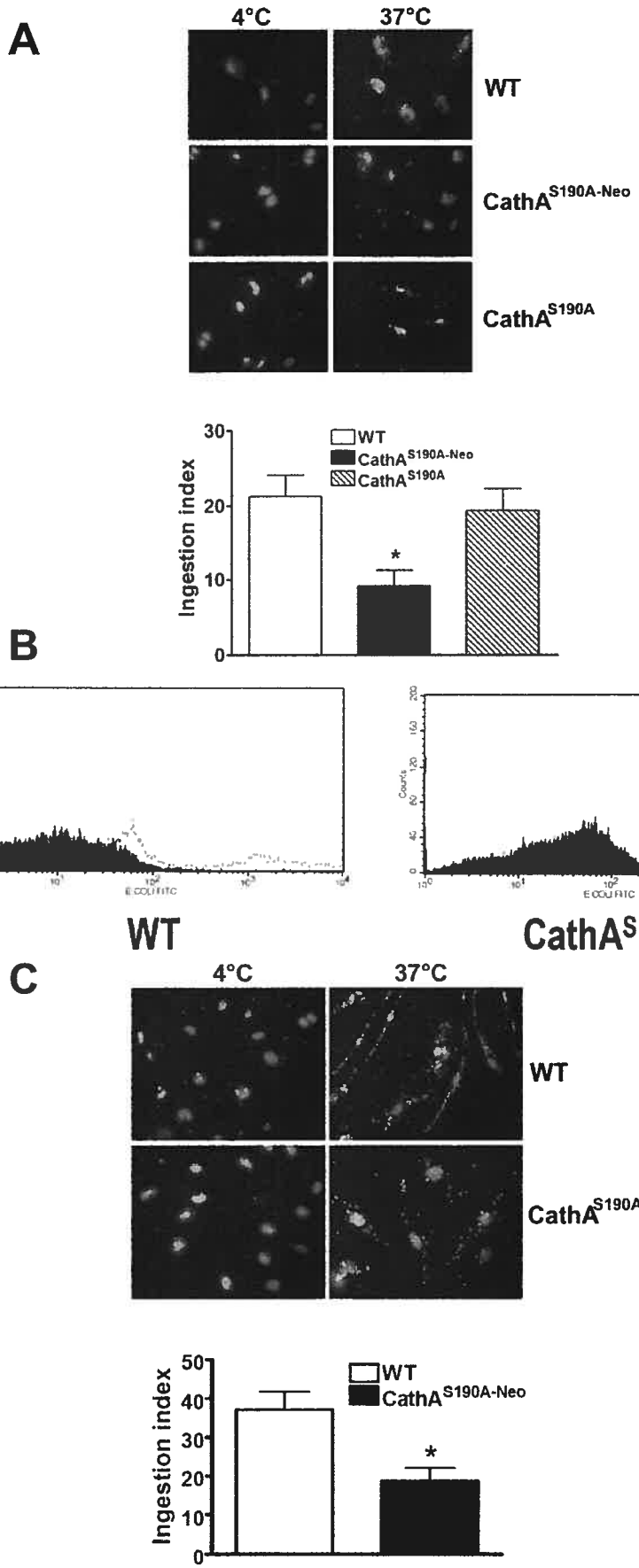

A
4°C
37°C
WT
CathA^S190A-Neo
CathA^S190A
Ingestion index
30
20
10
0
WT
CathA^S190A-Neo
CathA^S190A
*
B
E.COLI FITC
WT
CathA^S190A-Neo
C
4°C
37°C
WT
CathA^S190A-Neo
Ingestion index
50
40
30
20
10
0
WT
CathA^S190A-Neo
*

Figure 3

Figure 3. Reduced phagocytosis in *CathA*$^{S190A\text{-}Neo}$ mice splenocytes-derived macrophages and immature DC.

A. Splenocyte-derived macrophages from the WT, *CathA*$^{S190A\text{-}Neo}$ and *CathA*^{S190A} mice were incubated for 3 h at 4°C or at 37°C in the presence of fluorescein-labeled *E. coli* bioparticles (50 particles per one cell), fixed, and stained with DAPI. Slides were examined on the Nikon Eclipse E6000 direct epifluorescence microscope. Magnification 200x. Panels represent typical images obtained in triplicate experiments; from 70 to 150 cells (10 panels) were studied for each condition in each experiment. Ingestion index was calculated for each type of cells as the percentage of macrophages with phagocytized particles multiplied by the average number of particles phagocytized per ingested macrophage. The results show an average ± SD of 3 independent experiments. * $p< 0.01$.

B. The macrophages obtained from spleens of the WT (left panel) or the *CathA*$^{S190A\text{-}Neo}$ mice were incubated for 3 hrs with FITC-conjugated E. *coli* at 37°C or 4° C at 1:50 ratio, washed with PBS, fixed in 4% paraformaldehyde and analyzed by flow cytometry. The panels show overlap of the histograms of the cells incubated with (red line) and without (blue line) FITC-conjugated *E. coli*. The shift in the histograms on the left panel represents the cells that have engulfed the labeled *E. coli*.

C. Splenocyte-derived immature DC from the WT and *CathA*$^{S190A\text{-}Neo}$ mice were incubated for 3 h at 4°C or at 37°C in the presence of fluorescein-labeled *E. coli* bioparticles (50 particles per one cell), fixed, and stained with DAPI. Slides were examined on the Nikon Eclipse E6000 direct epifluorescence microscope. Magnification 200x. Panels represent typical images obtained in triplicate experiments; from 70 to 150 cells (10 panels) were studied for each condition in each experiment. Ingestion index was calculated as described for marcrophages. The results show an average ± SD of 3 independent experiments. * $p < 0.01$.

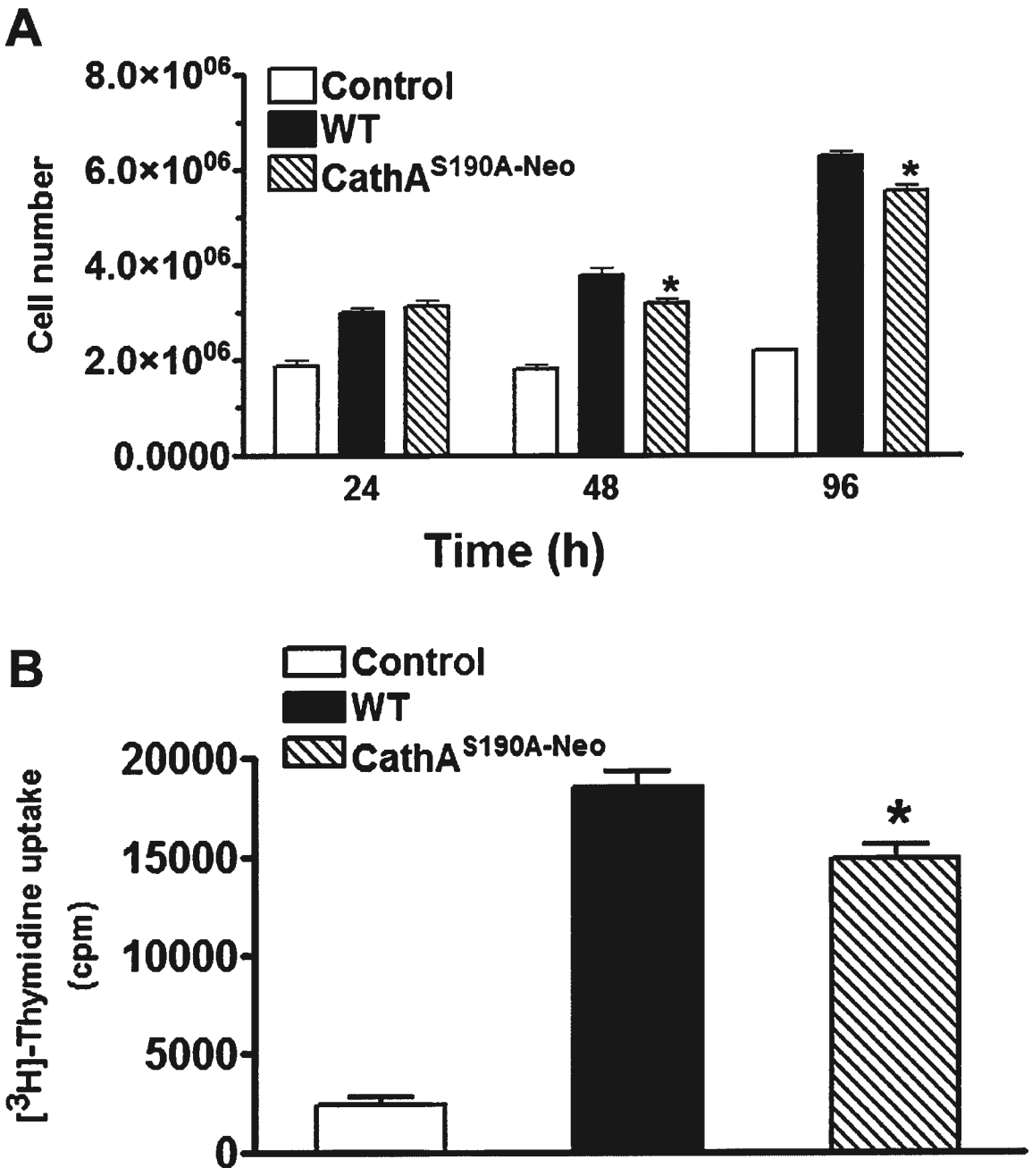

A
8.0×10^06
6.0×10^06
4.0×10^06
2.0×10^06
0.0000
Cell number
Control
WT
CathA S190A-Neo
*
*
24
48
96
Time (h)
B
Control
WT
CathA S190A-Neo
20000
15000
10000
5000
0
[3H]-Thymidine uptake
(cpm)
*

Figure 4

Figure 4. Proliferation rate of lymphocytes induced by the mature splenocyte-derived DC measured by cell counting (A) and [methyl-^{3}H]-thymidine incorporation (B).

A. Mice lymphocytes (2 x 10^6 in 3 ml medium) were incubated at 37°C in the presence or absence of 1 x 10^5 of mature splenocyte-derived DC. At the time intervals indicated on the figure the cells were counted using haemacytometer. Values represent means ± S.D. of triplicate experiments. * $p < 0.05$.

B. Mouse lymphocytes (2 x 10^6 in 3 ml medium) were incubated at 37°C in the presence or absence of 1 x 10^5 of mature splenocyte-derived DC in the medium containing [methyl-^{3}H]-thymidine. After 96 hrs the cells were harvested and the [^{3}H]-radioactivity was measured in cell homogenates by scintillation counting. The amounts of incorporated [methyl-^{3}H]-thymidine are shown as counts per minute. Values represent means ± S.D. of triplicate measurements. * $p< 0.05$.

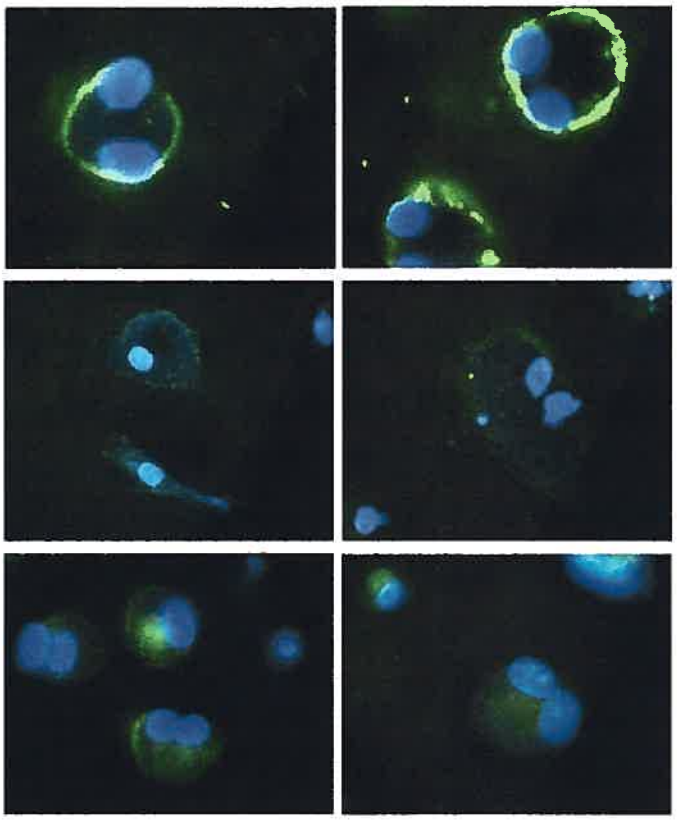


Figure 5

Figure 5. Induced cell surface staining of *CathA*$^{S190A\text{-}Neo}$ mice splenocytes-derived macrophages by the lectin from *Maackia amurensis*.

Splenocyte-derived macrophages from the WT, *CathA*$^{S190A\text{-}Neo}$ and *CathA*^{S190A} mice were incubated for 15 min at 37°C in the presence of fluorescein-labeled lectin from *Maackia amurensis* fixed, and stained with DAPI. Slides were examined on the Nikon Eclipse E6000 direct epifluorescence microscope. Magnification 1000x. Panels represent typical images obtained in triplicate experiments; at least 30 cells were studied for each condition in each experiment.

CHAPTER 5: GENERAL DISCUSSION AND CONCLUSION

5.1 General discussion

The role of the lysosomal sialidase Neu1 in the degradation of sialylated glycolipids and glycoproteins is well-established. However, previous works including those from our laboratory suggested that Neu1 is also involved in cellular signaling during the immune response (Chen et al., 1997; Wang et al., 2004, Lukong et al., 2001, Katoh et al., 1999). Since the innate immune system plays an important role in the defence against infectious agents and initiates inflammatory responses, knowledge of the biological role of the Neu1 in the immune cells should provide insights into the cellular immune response and may eventually lead to developing new approaches for therapeutic intervention.

Macrophages and DCs together called antigen-presenting cells (APCs) are unsurpassed in their ability to stimulate immunologically naive T cells. They recognize, phagocytize and process foreign objects (e.g. bacteria, viruses) and present antigens on the cell surface for stimulation of other cells of the immune system. Desialylation of cell surface glycoconjugates *in vivo* may make monocytes and macrophages more receptive to activation (Stamatos et al., 2004) and increase their chemotactic response to sites of inflammation, as was shown in PMNs (Cross et al., 2003). In particular, macrophages and DCs may be able to enhance the immunogenicity of processed antigens if the increased sialidase activity results in removal of the sialic acid masks of concealed epitopes (Benjouad et al., 1993).

The main goal of our project was to characterize the biological role of Neu1 in the APCs and in particular to clarify its role in cell signaling and during the cell maturation and activation.

1. Endogenous Neu1 activity is induced during the differentiation of monocytes into macrophages.

First, we have studied the expression level of Neu1 during the differentiation of freshly isolated human circulating peripheral blood monocytes. Circulating peripheral blood monocytes play a key role in potentiating diverse immune activities and can differentiate into either macrophages or DCs by exposure to specific stimuli (Sallusto et al., 1994). During the differentiation the function of monocytes changes from antigen recognition and processing to antigen presentation in macrophages and DCs. The second cell model that we have used was THP-1 cells, a monocytic cell line derived from the peripheral blood of a patient with acute monocytic leukemia (Tsuchiya et al., 1980). Being induced by 12-O-tetradecanoylphorbol-13-acetate (PMA) these cells differentiate into adherent cells having morphological, biochemical and physiological characteristics of macrophages and expressing the characteristic macrophage cell surface phenotypic markers, such as CD14 and CD206 (Auwerx et al., 1991). THP-1 cells have several advantages over primary human monocytes (higher transfection efficiency, homogeneity, accessibility) that make them a reliable model to study the mechanism of monocytic differentiation.

We have shown that Neu1 was upregulated during monocyte-to-macrophage differentiation in both models. As we have described in **article 1** and **article 2**, the endogenous sialidase activity of freshly isolated human monocytes increases 13- to 14-fold as cells differentiate *in vitro* into macrophages. It also increases 3- to 4-fold during the differentiation of THP-1 cells into THP-1-derived macrophages. The increase in the sialidase activity during the differentiation of monocyte-to-macrophage was consistent with the data observed during the activation of T cells by Chen and Landolfi (Chen et al., 2000 and Landolfi et al., 1985) and Lukong et al. (2001). The observed increase of the endogeneous sialidase activity,

however, could be caused by upregulation of any of 3 previously described human sialidases: lysosomal (Neu1, gene *NEU1* and Neu4, gene *NEU4*), cytoplasmic (Neu2, gene *NEU2*) and plasma membrane (Neu3, gene *NEU3*). Since the cytosolic sialidase Neu2 is mostly active at neutral pH (Monti et al., 1999), and the sialidase activity measured with 4 MU-NANA at pH 7.5 was not increased during the differentiation of THP-1 cells we were able to exclude this enzyme. The other 3 remaining enzymes, Neu1, Neu3 and Neu4 all have acidic pH optimum but they differ from each other in substrate specificity: Neu1 shows the highest activity against oligosacharides and short glycopeptides (Miyagi et al., 1993) whereas Neu3 and Neu4 are equally active against α2-3-sialylated oligosaccharides, glycopeptides and gangliosides (Schneider-Jakob et al., 1991). Sialidase activity of THP-1 cells measured against sialylated glycolipids (mixed bovine gangliosides) was slightly reduced during differentiation suggesting that the increase of sialidase activity is caused by the upregulation of the lysosomal sialidase Neu1.

Neu1 exists as a part of a multienzyme complex with β-D-galactosidase and CathA (Pshezhetsky et al., 2001, van der Horst et al., 1989, Tranchemontagne et al., 1990 and Galjart et al., 1991) and can be immunoprecipitated selectively from cell lysates using anti-CathA antibodies (Pshezhetsky et al., 1994), but neither Neu2 nor Neu3 or Neu4 form oligomeric structures when purified from tissues (Miyagi et al., 1990 and Miyagi et al., 1985). In our studies, we showed that more than 99% of the induced sialidase activity in macrophages was eliminated by anti-CathA antibodies providing the evidence that Neu1 was the source of the endogenous sialidase activity induced during the differentiation of monocytes into macrophages.

To confirm the Neu1 induction at the RNA level, quantitative RT-PCR and Northern blots were applied. Both methods showed that during the differentiation the *Neu1* mRNA was increased, whereas *Neu3* mRNA was decreased. Neu2 expression

could not be detected in the THP-1 monocytes as well as in the THP-1-derived macrophages neither by RT-PCR nor by Northern blots. Western blots with the polyclonal anti-Neu1 and anti-Neu3 antibodies confirmed the up-regulation of *Neu1* and downregulation of *Neu3* in THP-1 cells at the protein level. Interestingly, in the primary blood macrophages the amount of both *Neu1* and *Neu3* RNA detected by real time RT-PCR was ~3-fold increased as monocytes differentiated into macrophages but Western blot analysis using specific antibodies showed that the Neu1 protein was induced at much higher level then the Neu3 protein.

The transcriptional activity of the *NEU1* gene promoter was studied by deletion mapping. Five restriction fragments of the *NEU1* gene 5'-flanking region were cloned into a pBLCAT6 vector (Boshart et al., 1992) in front of a reporter *CAT* gene and expressed in COS-7 cells. We showed that the highest promoter activity was associated with a 542 bp fragment. Extension of this fragment to 936 bp and further to 1598 bp decreased the promoter activity, suggesting that potential elements for down-regulation of the gene expression could be present between the nucleotides -542 and -1598 (**article 2**). We further tested whether the promoter activity of the *NEU1* gene was induced during the differentiation of THP-1 cells into macrophages. We found that 24 h after the induction of cells with PMA, the CAT activity was significantly increased for all constructs, the highest effect (8-fold increase) being achieved for the reporter construct, pBL-NEU1CAT3 containing the 542 bp promoter fragment. The highest level of induction was observed for a 542 bp fragment of the *NEU1* gene promoter containing several potential sequence motifs for binding AP-1, a group of homodimeric and heterodimeric complexes composed of various *fos* and *jun* gene products, such as c-Jun, JunB, JunD, c-Fos, and FosB as well as the Fos-related antigens Fra-1 and Fra-2 (Cohen et al., 1989, Curran et al., 1988, Lee et al., 1987, Ransone et al., 1990, Rauscher et al., 1988 and Schonthal et al., 1988). All

of these complexes (also called protooncoproteins) interact with the common binding sites called PMA-responsive elements and mediate gene induction in response to growth factors, cytokines, phorbol esters, and a variety of other stimuli, including oxidative stress (Haliday, et al., 1991, Hanazawa et al., 1993 and Lo et al., 1995). In particular, one of the functions of AP-1 is to mediate inflammatory responses in lymphocytes and macrophages by stimulating cell activation and the expression of cytokines (Tanaka et al., 1997).

Altogether our data showed that the differentiation of monocytes to macrophages results in significant and specific induction of the *Neu1* gene.

2. Neu1 and CathA are targeted to the cell surface during the differentiation of monocytes to macrophages.

Our further studies described in **article 2** provided a clear evidence for the Neu1 translocation from the lysosomes to the cell surface during the immune cell activation and differentiation. The intracellular localization of Neu1 was studied by confocal immunomicroscopy. In the nondifferentiated cells, Neu1 and CathA showed a cytoplasmic punctate staining pattern co-localizing with the lysosomal marker LAMP-2. In contrast, in the differentiated cells the majority of Neu1 and CathA was found on the cell periphery, whereas LAMP-2 still had the intracellular localization (**article 2**). We speculate that in the differentiating cells both enzymes are first targeted to the endosomal/lysosomal compartment, where they gain enzymatic activity and then are sorted to the vesicles destined to the plasma membrane. As discussed above, in the lysosome, Neu1 is a component of a multienzyme complex, with CathA, ß-galactosidase and N-acetylgalactosamine-6-sulfate sulfatase (Pshezhetsky et al., 2001). If the complex is disrupted, Neu1 loses its enzymatic

activity and is rapidly degraded by the lysosomal proteases (van der Horst et al., 1989 and Manzi et al., 1994). CathA, as the majority of soluble lysosomal lumenal proteins, is targeted through the mannose-6-phosphate receptor pathway (Lukong et al., 1999), whereas the intracellular trafficking of Neu1 is mediated by the adaptor proteins through a Tyr-containing motif in its C-terminus (Lukong et al., 2001). The two proteins meet and undergo activation in the lysosome. The activation of CathA involves its processing by a lysosomal endopeptidase which removes a 2-kDa "linker" polypeptide between the two polypeptide chains of the mature enzyme (Bonten et al., 1995). Although the exact molecular mechanism of the Neu1 activation is unknown but most likely it involves a conformational change of the Neu1 polypeptide after it forms the complex with CathA. It is possible therefore that lysosomal Neu1 and CathA may travel to the cell surface as a complex inside the exocytic vesicles that fuse with the cell membrane. The existence of such lysosome-to-plasma membrane transport pathway has been well documented for other professional APCs, DCs where it plays a crucial role in targeting the peptide-MHC II complexes (pMHCs) to the cell surface (Turley et al., 2000). Upon receipt of a maturation stimulus (such as lipopolysacharide) the internalized antigens are processed in the endosomes and pMHCs are formed. Several hours later they appear in the LAMP-negative compartments termed CIIV, which are the intermediates in the transfer of pMHCs to the plasma membranes (Turley et al., 2000). The concentration of pMHCs in CIIV reaches a maximum 12 hours after the lipopolysacharide treatment and after 12 more hours the majority of complexes is already transferred to the cell surface. The described targeting of pMHCs in DCs resembles the targeting of Neu1 and CathA in differentiating monocytes and macrophages. We showed (**article 2**) that during the differentiation of monocytes into macrophages the Neu1 is constitutively transported to the plasma membrane along

with class II molecules rather then being actively retained in the lysosomes. We found that 12 to 24 hours after receiving the differentiation stimulus the lysosomal-endosomal apparatus of monocytes undergoes a dramatic rearrangement. The MHC II-positive and Neu1-positive compartments are segregated from the LAMP-2 positive lysosomes and late endosomes, relocated closer to the cell surface and fused with the plasma membranes. The same vesicles are stained positively for the internalized antigen horseradish peroxidase (HRP) or fluorescent dextran, which in the non-differentiated monocytic cells are normally stored for at least several days in LAMP-2-positive endosomal/lysosomal compartment. In the differentiating cells the dextran molecules and HRP peptide (probably in the form of pMHCs) were gradually accumulated on the cell plasma membrane colocalizing at least in part with Neu1 and CathA.

Although the exact targeting mechanism of Neu1 and CathA still has to be clarified, the data we obtained showed that the most possible pathway is that Neu1 and CathA are being sorted from the Golgi to the lysosomes and then to the LAMP-2-negative, MHC II-positive vesicles, which later merge with the plasma membrane of immune cells during their differentiation or activation. Most recently, Nan et al. (2007) showed that the complex between CathA and Neu1 is also detected on the surface of activated T cells (Nan et al., 2007), which is in a good agreement with our studies. The identified re-localization of Neu1 during the differentiation of monocytes not only provides essential information about the enzyme's biogenesis but also may provide a clue about its role in the immune cells. Our hypothesis is that Neu1 on the cell surface may be involved in the desialylation of membrane proteins and therefore in the modulation of the cell activity.

3. Suppression of the Neu1 expression in macrophages alters their functional capacity

In order to understand whether upregulation of the Neu1 expression and its targeting to the cell surface is important for the primary function of macrophages we have used siRNA to inhibit the expression of Neu1 in THP-1 cells. We found that the suppression of Neu1 expression in the THP-1-derived macrophages by siRNA or their treatment with anti-Neu1 antibodies significantly reduced functional capacity of the cells, such as their ability to engulf bacteria or to produce cytokines (**article 2**), suggesting that the upregulation of the Neu1 expression is important for activation of macrophages and establishing the link between Neu1 and cellular immune response. Recently, it was also found that Neu1 is important for the production of interferon gamma by T cells (Nan et al., 2007).

4. Neu1 sialidase but not CathA activity on the surface of the immune cells is important for the functional integrity.

Activation and stabilization of the Neu1 in the lysosome, requires its association with the lysosomal multienzyme complex (LMC) also containing the lysosomal CathA, ß-galactosidase and N-acetylgalactosamine-6-sulfate sulfatase. Furthermore we already identified that Neu1 on the surface of macrophages is tightly associated with CathA during the cell differentiation (**article 2**). Interestingly, during the differentiation of macrophages, only CathA but not other components of LMC is sorted to the plasma membrane similarly to Neu1. Both proteins were first targeted to the lysosome and then sorted to LAMP-2-negative, MHC II-positive vesicles, later merged with the plasma membrane. Therefore, we decided to investigate if CathA activity on the cell surface is also required for the immune function.

Recently, animal models of single CathA deficiency and double CathA/Neu1 deficiency have been developed in our laboratory by gene targeting in mice. In the first line of mice (*CathA*^{S190A}) we have replaced the nucleophyl of the CathA active site, Ser190 with Ala, which abolished the enzymatic activity, while the mutant protein retained its ability to activate Neu1. In addition to *CathA*^{S190A} mice we have produced a line (*CathA*$^{S190A\text{-}Neo}$ mice) that had a *PGK-neo* cassette inserted in the *CathA* gene. These mice had a significantly reduced (3-10%) Neu1 activity, due to dramatically decreased *CathA* mRNA levels, consistent with previously reported hypomorphic effects of the *neo* gene (Seyrantepe et al., in preparation). Therefore, we have obtained hypomorphic CathA mice with the secondary deficiency of the Neu1 activity. Since the residual level of the Neu1 activity in the tissues of the *CathA*$^{S190A\text{-}Neo}$ mice is still higher than that in the previously described knockout CathA mice (Zhou et al., 1995), the animals do not develop severe galactosialidosis phenotype. Therefore, the described above mouse models provided us with a possibility to study the impact of the Neu1 and CathA deficiency on the function of different types of the immune cells *ex vivo*.

Our data (**article 3**) strongly indicated that the immune cells from the Neu1/CathA-deficient CathA$^{S190A\text{-}Neo}$ mice but not from the CathA-deficient CathA^{S190A} mice have significantly reduced functional capacity. First we found that the splenocyte- and PBMC-derived macrophages, the peritoneal adherent cells as well as the splenocyte-derived immature DCs from the CathA$^{S190A\text{-}Neo}$ mice showed reduced ability to phagocytose pathogens, whereas the cells from the CathA^{S190A} mice had normal properties. This in turn could mean that these cells are less efficiently activated, which is consistent with our previous observation that the THP-1-derived macrophages showed reduced production of cytokines including IL-1b, IL-6 and TNF-α after the Neu1 activity on the their surface was inhibited with the anti-Neu1

antibodies (**article 2**). Furthermore, the mature DCs from the Neu1-deficient mice showed reduced ability to activate the proliferation of T lymphocytes. In addition, the macrophages from the Neu1-deficient mice showed higher staining with the FITC conjugated *Maackia amurensis* lectin (**article 3**) suggesting that they have increased sialylation of the cell surface glycoproteins. Our data suggest, therefore, that the cell surface Neu1 plays an important role in the regulation of affinity of the immune cells towards each other and external pathogens. They also provide an evidence that Neu1 but not CathA on the surface of immune cells plays a key role in the regulation of their functional capacity.

In conclusion, our results indicate that the lysosomal sialidase Neu1 plays an essential role in the removal of terminal sialic acid residues from the cell surface glycoproteins and is important for activation of the immune cells. Previous studies have identified on the surface of macrophages sialic acid binding receptors (siglecs), such as sialoadhesin (Sn), CD22, myelin-associated glycoprotein, CD33, and siglec-5 and showed that they are involved in discrete functions in the hemopoietic, immune, and nervous systems (Munday et al., 1999). The expression of siglecs is highly regulated and normally only found at high levels on distinct subsets of tissue macrophages (Crocker et al., 1986, 1994). The molecular features and binding properties of siglecs suggest that they evolved as an accessory molecule to promote interactions of macrophages with host cells and extracellular matrix displaying appropriate sialylated ligands. Characterization of cells that bind to siglecs has revealed high binding of granulocytes (Crocker et al., 1995) and lymphoma cells (van den Berg et al., 1992). Macrophage-host cell interactions that could be influenced by siglecs, also include antigen presentation to primed T cells (Muerkoster et al., 1999) and recognition and clearance of apoptotic cells, effete erythrocytes, and opsonized cells (e.g., in malaria and certain autoimmune diseases). To date, all sialic

acid-dependent binding of macrophages has been found to be siglec-dependent. This would suggest that on macrophages, siglec is the major sialic acid binding receptor responsible for mediating cell-cell interactions and that the primary function of other siglecs may be in signaling. Therefore the optimal sialylation of the cell surface glycoproteins including siglecs themselves should play the key role in the interaction of macrophages with other cells including T, B and NK cells. The marked expansion of CD33-related siglecs in the innate immune system suggests that these proteins play also a fundamentally important role in host-defense functions since sialic acids are also present on the surface of many pathogens.

Since desialylation of siglecs significantly increases their affinity towards sialic acid, our data suggest the mechanism by which the cell surface Neu1 regulates the affinity of the immune cells towards each other and external pathogens. Therefore it is tempting to speculate that similarly to CD63, CD152, CD206 and other lysosomal/endosomal proteins expressed on the surface of the immune cells, Neu1 is important for their functional integrity.

Together with previously obtained data on the importance of the Neu1 for the IL4 production by the lymphocytes (Chen et al., 1997, 2000) our results show that the upregulation of Neu1 sialidase plays an important role in signaling of immune cells by regulating the cytokine production and suggest that in human sialidosis patients Neu1 deficiency may result in reduced capacity of immune cells to produce cytokines and antibodies leading to partial immunodeficiency and may account for frequent pulmonary infections. Further experiments are necessary to clarify if the differentiation-induced translocation of the lysosomal sialidase, Neu1 and CathA to the plasma membrane is also important for other specific functions of the immune cells and, in particular, for the antigen presentation. However, since fully differentiated macrophages are present in all body tissues (microglial cells in the CNS,

Kupffer cells in liver, etc.) it is quite conceivable that the primary or secondary deficiency of the Neu1 activity may affect functions of all monocyte-derived cells and cause defects in multiple tissues and organs.

Altogether our results reveal important role of the Neu1 sialidase in immune response and cell signaling and bring new insight into the biogenesis and function of antigen presenting cells.

5.2 Conclusions

1. Expression of the lysosomal sialidase Neu1 is upregulated during the differentiation of monocytes into macrophages and DCs as well as during the maturation of DCs, due to a significant induction of the transcriptional activity of the *Neu1* gene promoter.

2. During the differentiation of macrophages Neu1 together with its activator protein, CathA is first targeted to the lysosome and then sorted to the LAMP-2-negative, MHC II-positive vesicles, which later merge with the plasma membrane.

3. Suppression of the Neu1 expression in the macrophages reduces their ability to produce cytokines.

4. Macrophages and DCs derived from the hypomorphic CathA mice with the secondary ~90% reduction of the Neu1 activity show significantly increased sialylation of the surface, and dramatically reduced capacity to engulf bacteria, whereas the mature DCs have lower effect on T cells proliferation.

5. Altogether our data suggest that Neu1 regulates the affinity of the immune cells towards each other and towards external pathogens and is therefore necessary for their functional integrity.

REFERENCES

Akita H, T. Miyagi, K. Hata, and M. Kagayama. Immunohistochemical evidence for the existence of rat cytosolic sialidase in rat skeletal muscles. *Histochem. Cell. Biol.* 107(6), 495-503 (1997).

Andria, G., G. Strisciuglio, W.S. Pontarelli, W.S. Sly, and W.E. Dodson. *In "Sialidases and Sialidoses"* (P. Durand et al., eds,) p.365., Edi. Ermes, Milano, (1981).

Auwerx J. The human leukemia cell line, THP-1: a multifacetted model for the study of monocyte-macrophage differentiation. *Experientia.* 47(1):22-31 (1991).

Bame, K. J. and Rome, L. H. Acetyl coenzyme A:alpha-glucosaminide N-acetyltransferase: evidence for a transmembrane acetylation mechanism. *J. Biol. Chem.* 260, 11293-11299 (1985).

Bao M, Booth JL, Elmendorf BJ, Canfield WM Bovine UDP-N-acetylglucosamine:lysosomal-enzyme N-acetylglucosamine-1-phosphotransferase. I. Purification and subunit structure. *J Biol Chem.* 271(49), 31437-45 (1996).

Barriocanal J.G., Bonifacino J.S., Yuan L., Sandoval I.V. Biosynthesis, glycosylation, movement through the Golgi system, and transport lysosomes by an N-linked carbohydrate-independent mechanism of three lysosomal integral membrane proteins. *J Biol Chem.* 261(35), 16755-63 (1986).

Benjouad A, Babas T, Montagnier L, Bahraoui E. N-linked oligosaccharides of simian immunodeficiency virus envelope glycoproteins are dispensable for

the interaction with the CD4 receptor. *Biochem Biophys Res Commun.* 190(2):311-9 (1993).

Bonten, E.J., N.J. Galjart, R. Willemsen, M. Usmany, J.M. Vlak, and A. d'Azzo. Lysosomal protective protein/cathepsin A. Role of the "linker" domain in catalytic activation. *J. Biol. Chem.* 270, 26441-26445 (1995).

Bonten, E.J., A. van der Spoel, M. Fornerod, G. Grosveld, and A. d'Azzo Characterization of human lysosomal neuraminidase defines the molecular basis of the metabolic storage disorder sialidosis. *Genes and Devel.* 10, 3156-3168 (1996).

Le Borgne R. and Hoflack B. Protein transport from the secretory to the endocytic pathwayin mammalian cells. *Biochim Biophys Acta.* 1404(1-2), 195-209 (1998).

Boshart M, Kluppel M, Schmidt A, Schutz G, Luckow B. Reporter constructs with low background activity utilizing the cat gene. *Gene.* 110(1):129-30 (1992).

Bradshaw JM, Mitaxov V, Waksman G. Investigation of phosphotyrosine recognition by the SH2 domain of the Src kinase. *J Mol Biol.* 293(4), 971-85 (1999).

Braun M., Waheed A., von Figura K. Lysosomal acid phosphatase is transported to lysosomes via the cell surface. *EMBO J.* 8(12), 3633-40 (1989).

Cantz, M., J. Gehler, and J. Spranger. Mucolipidosis I: increased sialic acid content and deficiency of an alpha-N-acetylneuraminidase in cultured fibroblasts. *Biochem. Biophys. Res. Commun.*74, 732-738 (1977).

Carrillo, M.B., C.N. Milner, S.T. Ball, M. Snock, and R.D. Campbell. Cloning and characterization of sialidase from the murine histocompatibility-2 complex: low levels of mRNA and a single amino acid mutation are responsible for reduced sialidase activity in mice carrying the Neu1 allele. *Glycobiology* 7, 975-986 (1997).

Chen XP, E.Y. Enioutina, and R.A. Daynes The control of IL-4 gene expression in activated murine T lymphocytes: a novel role for neu-1 sialidase. *J. Immunol.* 158(7), 3070-3080 (1997).

Chen XP, X. Ding, and R.A. Daynes. Ganglioside control over IL-4 priming and cytokine production in activated t cells. *Cytokine* 12(7), 972-985 (2000).

Chikuma S, Murakami M, Tanaka K, Uede T. Janus kinase 2 is associated with a box 1-like motif and phosphorylates a critical tyrosine residue in the cytoplasmic region of cytotoxic T lymphocyte associated molecule-4. *J Cell Biochem.* 78(2), 241-50 (2000).

Chou MY, Li SC, Li YT. Cloning and expression of sialidase L, a NeuAcalpha2-->3Gal-specific sialidase from the leech, Macrobdella decora. *J Biol Chem.* 271(32), 19219-24 (1996).

Cohen DR, Ferreira PC, Gentz R, Franza BR Jr, Curran T. The product of a fos-related gene, fra-1, binds cooperatively to the AP-1 site with Jun: transcription factor AP-1 is comprised of multiple protein complexes. *Genes Dev.* 3(2):173-84 (1989).

Collawn J.F., Kuhn L.A., Liu L..F, Tainer J.A., Trowbridge I.S. Transplanted LDL and mannose-6-phosphate receptor internalization signals promote high-efficiency endocytosis of the transferrin receptor. *EMBO J.* 11, 3247-53 (1991).

Colman P.M. Influenza virus neuraminidase: structure, antibodies, and inhibitors. *Protein Sci.* 3(10), 1687-96 (1994).

Conzelmann E, Sandhoff K. Activator proteins for lysosomal glycolipid hydrolysis. *Methods Enzymol.* 138, 792-815 (1987).

Corfield, T. Bacterial sialidases--roles in pathogenicity and nutrition. *Glycobiology* 2, 509-521 (1992).

Crennell, S.J., E.F. Garman, W.G. Laver, E.R. Vimr, and G. Taylor. Crystal structure of a bacterial sialidase (from Salmonela typhimurium LT2) shows the same fold as an influenza virus neuraminidase. *Proc. Natl. Acad. Sci. USA* 90, 9852-9856 (1993).

Cretin H. The proton gradient across the vacuo-lysosomal membrane of lutoids from the latex of Hevea brasiliensis. I. Further evidence for a proton-translocating ATPase on the vacuo-lysosomal membrane of intact lutoids. *J Membr Biol.* 65(3), 175-84 (1982).

Crocker PR, Mucklow S, Bouckson V, McWilliam A, Willis AC, Gordon S, Milon G, Kelm S, Bradfield P. Sialoadhesin, a macrophage sialic acid binding receptor for haemopoietic cells with 17 immunoglobulin-like domains. *EMBO J.* 13(19):4490-503 (1994).

Crocker PR, Gordon S. Properties and distribution of a lectin-like hemagglutinin differentially expressed by murine stromal tissue macrophages. *J Exp Med.* 164(6):1862-75 (1986).

Crocker PR, Freeman S, Gordon S, Kelm S. Sialoadhesin binds preferentially to cells of the granulocytic lineage. *J Clin Invest.* 95(2):635-43 (1995).

Cross AS, Sakarya S, Rifat S, Held TK, Drysdale BE, Grange PA, Cassels FJ, Wang LX, Stamatos NM, Farese A, Casey D, Powell J, Bhattacharjee AK, Kleinberg M & Goldblum SE. Recruitment of murine neutrophils in vivo through endogenous sialidase activity. *J Biol Chem* 278, 4112–4120 (2003).

Curran T, Franza BR Jr. Fos and Jun: the AP-1 connection. *Cell.* 55(3):395-7 (1988).

d'Azzo, A., G. Andria, P. Strisciuglio, and H. Galijaard. Galactosialidosis. *In* Metabolic *and Molecular Bases of Inherited Disease*, (Scriver C.R., Beaudet A.L, Sly W.S. and Valle D., eds.) New York: McGraw-Hill, pp. 2835-2837 (1995).

d'Azzo,A., A. Hoogeveen, A.J. Reuser, D. Robinson, and H. Galjaard. Molecular defect in combined -galactosidase and neuraminidase deficiency in man. *Proc. Natl. Acad. Sci. U.S.A.* 79, 4535-4539 (1982).

Dahms NM, Lobel P, Kornfeld S. Mannose 6-phosphate receptors and lysosomal enzyme targeting. *J Biol Chem.* 264(21), 12115-8 (1989).

Dorland L, Haverkamp J, Viliegenthart JF, Strecker G, Michalski JC, Fournet B, Spik G, Montreuil J. 360-MHz 1H nuclear-magnetic-resonance spectroscopy of sialyl-oligosaccharides from patients with sialidosis (mucolipidosis I and II). *Eur J Biochem.* 87(2), 323-9 (1978).

Durand, P., R. Gatti, S. Cavalieri, C. Borrone, M. Tondeur, J.C. Michalski, and C. Strecker. Sialidosis (mucolipidosis I). *Helvetica Paediatrica Acta,* 32, 391-400 (1977).

Elsliger M.A., Potier M. Homologous modeling of the lysosomal protective protein/carboxypeptidase L: structural and functional implications of mutations identified in galactosialidosis patients. *Proteins.* 18(1), 81-93 (1994).

Erickson A.H., Conner G.E., Blobel G. Biosynthesis of a lysosomal enzyme. Partial structure of two transient and functionally distinct NH2-terminal sequences in cathepsin D. *J Biol Chem.* 256(21), 11224-31 (1981).

Federico, A., A. Cecio, G.A. Battini, J.C. Michalski, G. Strecker, and G.C. Guazzi. Macular cherry-red spot and myoclonus syndrome. Juvenile form of sialidosis *J. Neurol. Sci.* 48, 157-169 (1980).

Fingerhut R, G.T. van der Horst, F.W. Verheijen, and E. Conzelmann. Degradation of gangliosides by the lysosomal sialidase requires an activator protein. *Eur. J. Biochem.* 208(3), 623-629 (1992).

Frisch A, Neufeld EF. A rapid and sensitive assay for neuraminidase: application to cultured fibroblasts. *Anal Biochem.* 95(1), 222-7 (1979).

Fukuhara, Y.; Takano, T.; Shimmoto, M.; Oshima, A.; Takeda, E.; Kuroda, Y.; Sakuraba, H.; Suzuki, Y.: A new point mutation of protective protein gene in two Japanese siblings with juvenile galactosialidosis. *Brain Dysfunction* 5, 319-325, (1992).

Galen,J.E. *et al.* Role of *Vibrio cholerae* neuraminidase in the function of cholera toxin. *Infect. Immun.*, 60, 406-415 (1992).

Galjaard, H., A.T. Hoogeveen, H.A. de Wit-Verbeek, A.J.J. Reuser, M.W. Ho, and D. Robinson. *Nature* 257, 60-62 (1975).

Galjart, N.J., N. Gillemans, A. Harris, T. Gijsbertus, J. van der Horst, F.W. Verheijen, H. Galjaard, and A. d'Asso. Expression of cDNA encoding the human "protective protein" associated with lysosomal -galactosidase and neuraminidase: homology to yeast proteases. *Cell* 54, 755-764 (1988).

Galjart, N.J., H. Morreau, R. Willemsen, N. Gillemans, E.J. Bonten, and A. d'Azzo. Human lysosomal protective protein has cathepsin A-like activity distinct from its protection function. *J. Biol. Chem.* 266, 14754-14762 (1991).

Gee, K., Kozlowski, M., and Kumar, A. Tumor necrosis factor-alpha induces functionally active hyaluronan-adhesive CD44 by activating sialidase through p38 mitogen-activated protein kinase in lipopolysaccharide-stimulated human monocytic cells. *J Biol Chem.* 278(39), 37275-87 (2003).

Goldberg, M. F.; Cotlier, E.; Fichenscher, L. G.; Kenyon, K. R.; Enat, R.; Borowsky, S. A.: Macular cherry-red spot, corneal clouding, and beta-galactosidase deficiency: clinical, biochemical, and electron microscopic study of a new autosomal recessive storage disease. *Arch. Intern. Med.* 128, 387-398 (1971).

Gottschalk,A. and P.E. Lind. Product of interaction between influenza virus enzyme and ovomucin. *Nature* 164, 232-233 (1989).

Guarnieri F.G., Arterburn L.M., Penno M.B., Cha Y., August J.T. The motif Tyr-X-X-hydrophobic residue mediates lysosomal membrane targeting of lysosome-associated membrane protein 1. *J Biol Chem* 268(3), 1941-6 (1993).

Hanazawa S, Takeshita A, Amano S, Semba T, Nirazuka T, Katoh H, Kitano S. Tumor necrosis factor-alpha induces expression of monocyte chemoattractant JE via fos and jun genes in clonal osteoblastic MC3T3-E1 cells. *J Biol Chem.* 268(13):9526-32 (1993).

Haliday EM, Ramesha CS, Ringold G. TNF induces c-fos via a novel pathway requiring conversion of arachidonic acid to a lipoxygenase metabolite. *EMBO J.* 10(1):109-15 (1991).

Havelaar AC, Beerens CE, Mancini GM, Verheijen FW. Transport of organic anions by the lysosomal sialic acid transporter: a functional approach towards the gene for sialic acid storage disease. *FEBS Lett.* 446(1), 65-8 (1999).

Hers, H.G. The role of the pathogeny of storage diseases. *Biochimie* 54, 753-757 (1972).

Hinek A Biological roles of the non-integrin elastin/laminin receptor *Biol. Chem.* 377(7-8), 471-480 (1996).

Hiraiwa M, M. Nishizawa, Y. Uda, T. Nakajima, and T. Miyatake. Human placental sialidase: further purification and characterization. *J. Biochem. (Tokyo)* 103(1), 86-90 (1988).

Hiraiwa M, Y. Uda, M. Nishizawa, and T. Miyatake. Human placental sialidase: partial purification and characterization. *J. Biochem. (Tokyo)* 101(5), 1273-1279 (1987).

Hirst J. and M.S. Robinson. Clathrin and adaptors. *Biochim. Biophys. Acta* 1404(1-2), 173-193 (1998).

Hoflack B. and Kornfeld S. Lysosomal enzyme binding to mouse P388D1 macrophage membranes lacking the 215-kDa mannose 6-phosphate receptor: evidence for the existence of a second mannose 6-phosphate receptor. *Proc Natl Acad Sci U S A* 82(13), 4428-32 (1985).

Hoogeveen A, A. d'Azzo, R. Brossmer, and H. Galjaard. Correction of combined beta-galactosidase/neuraminidase deficiency in human fibroblasts. *Biochem. Biophys. Res. Commun.* 103(1), 292-300 (1981).

Hoogeveen AT, F.W. Verheijen, A. d'Azzo, and H. Galjaard. Genetic heterogeneity in human neuraminidase deficiency. *Nature* 285(5765), 500-502 (1980).

Hoogeveen, A.T., F.W. Verheijen, and H. Galjaard. The relation between human lysosomal -galactosidase and its protective protein. *J. Biol. Chem.* 258, 12143-12146 (1983).

Hrebicek M, Mrazova L, Seyrantepe V, Durand S, Roslin NM, Noskova L, Hartmannova H, Ivanek R, Cizkova A, Poupetova H, Sikora J, Urinovska J, Stranecky V, Zeman J, Lepage P, Roquis D, Verner A, Ausseil J, Beesley CE, Maire I, Poorthuis BJ, van de Kamp J, van Diggelen OP, Wevers RA, Hudson TJ, Fujiwara TM, Majewski J, Morgan K, Kmoch S, Pshezhetsky AV. Mutations in TMEM76* cause mucopolysaccharidosis IIIC (Sanfilippo C syndrome). *Am J Hum Genet.* 79(5), 807-19 (2006).

Hubbes M, D'Agrosa RM, Callahan JW. Human placental beta-galactosidase. Characterization of the dimer and complex forms of the enzyme. *Biochem J.* 285 (Pt 3), 827-31 (1992).

Huang JQ, J.M. Trasler, S. Igdoura, J. Michaud, N. Hanal, and R.A. Gravel. Apoptotic cell death in mouse models of GM2 gangliosidosis and observations on human Tay-Sachs and Sandhoff diseases. *Hum. Mol. Genet.* 6(11), 1879-1885 (1997).

Hunziker W., Geuze H.J. Intracellular trafficking of lysosomal membrane proteins. *Bioessays.* 18(5), 379-89 (1996).

Igdoura SA, C. Mertineit, J.M. Trasler, and R.A. Gravel. Sialidase-mediated depletion of GM2 ganglioside in Tay-Sachs neuroglia cells. *Hum. Mol. Genet.* 8(6), 1111-1116 (1999).

Igdoura, S.A., C. Gafuik, C. Mertineit, F. Saberi, A.V. Pshezhetsky, M. Potier, J.M. Trasier, and R.A. Gravel. Cloning of the cDNA and the gene encoding mouse lysosomal sialidase and correction of sialidase deficiency in human sialidosis and mouse SM/J fibroblasts. *Hum. Mol. Genet.* 7, 115-121 (1998).

Ikehara Y, Ikehara SK, Paulson JC. Negative regulation of T cell receptor signaling by Siglec-7 (p70/AIRM) and Siglec-9. *J Biol Chem.* 2279(41):43117-25 (2004).

Irani DN, Lin KI, Griffin DE. Brain-derived gangliosides regulate the cytokine production and proliferation of activated T cells. *J Immunol.* 157(10):4333-40 (1996).

Kanda N, Nakai K, Watanabe S. Gangliosides GD1b, GT1b, and GQ1b suppress the growth of human melanoma by inhibiting interleukin-8 production: the inhibition of adenylate cyclase. *J Invest Dermatol.* 117(2):284-93 (2001).

Kaplan A., Fischer D., Achord D, Sly W. Phosphohexosyl recognition is a general characteristic of pinocytosis of lysosomal glycosidases by human fibroblasts. *J Clin Invest.* 60(5), 1088-93 (1977).

Kato K, Shiga K, Yamaguchi K, Hata K, Kobayashi T, Miyazaki K, Saijo S, Miyagi T. Plasma-membrane-associated sialidase (NEU3) differentially regulates integrin-mediated cell proliferation through laminin- and fibronectin-derived signalling. *Biochem J.* 394(Pt 3):647-56 (2006).

Katoh S, Miyagi T, Taniguchi H, Matsubara Y, Kadota J, Tominaga A, Kincade PW, Matsukura S, Kohno S. Cutting edge: an inducible sialidase regulates the hyaluronic acid binding ability of CD44-bearing human monocytes. *J Immunol.* 162(9), 5058-61 (1999).

Kelly, T.E. and G. Graetz. Isolated acid neuraminidase deficiency: a distinct lysosomal storage disease. *Am. J. Med. Genet.,* 1, 31-46 (1977).

Kornfeld R., Bao M., Brewer K., Noll C., Canfield W. Molecular cloning and functional expression of two splice forms of human N-acetylglucosamine-1-phosphodiester alpha-N-acetylglucosaminidase. *J Biol Chem.* 274(46), 32778-85 (1999).

Kopitz J, C. von Reitzenstein, K. Sinz, and M. Cantz. Selective ganglioside desialylation in the plasma membrane of human neuroblastoma cells. *Glycobiology* 6(3), 367-376 (1996).

Kopitz J., Muhl C., Ehemann V., Lehmann C., Cantz M. Effects of cell surface ganglioside sialidase inhibition on growth control and differentiation of human neuroblastoma cells. *Eur J Cell Biol.* 73(1), 1-9 (1997).

Landolfi NF, J. Leone, J.E. Womack, and R.G. Cook. Activation of T lymphocytes results in an increase in H-2-encoded neuraminidase. *Immunogenetics* 22(2), 159-67 (1985).

Landolfi NF and R.G. Cook. Activated T-lymphocytes express class I molecules which are hyposialylated compared to other lymphocyte populations. *Mol. Immunol.* 23(3), 297-309 (1986).

Lee W, Mitchell P, Tjian R. Purified transcription factor AP-1 interacts with TPA-inducible enhancer elements. *Cell.* 49(6):741-52 (1987).

Leroy, J. G.; Demars, R. I. Mutant enzymatic and cytological phenotypes in cultured human fibroblasts. *Science* 157, 804-806 (1967).

Lin TJ, Garduno R, Boudreau RT, Issekutz AC. Pseudomonas aeruginosa activates human mast cells to induce neutrophil transendothelial migration via mast cell-derived IL-1 alpha and beta. *J Immunol.* 169(8):4522-30 (2002).

Loonen M.C., Lugt L, Franke C.L. Letter: Angiokeratoma corporis diffusum and lysosomal enzyme deficiency. *Lancet.* 2(7883); 785 (1974).

Lowden, J. A.; O'Brien, J. S.: Sialidosis: a review of human neuraminidase deficiency.*Am. J. Hum. Genet.* 31; 1-18 (1979).

Lo YY, Cruz TF. Involvement of reactive oxygen species in cytokine and growth factor induction of c-fos expression in chondrocytes. *J Biol Chem.* 270(20):11727-30 (1995).

Lukong KE, Elsliger MA, Mort JS, Potier M, Pshezhetsky AV. Identification of UDP-N-acetylglucosamine-phosphotransferase-binding sites on the lysosomal proteases, cathepsins A, B, and D. *Biochemistry.* 38(1):73-80 (1999).

Lukong KE, Elsliger MA, Chang Y, Richard C, Thomas G, Carey W, Tylki-Szymanska A, Czartoryska B, Buchholz T, Criado GR, Palmeri S, Pshezhetsky AV. Characterization of the sialidase molecular defects in sialidosis patients suggests the structural organization of the lysosomal multienzyme complex. *Hum Mol Genet.* 9(7),1075-85 (2000).

Lukong KE, Seyrantepe V, Landry K, Trudel S, Ahmad A, Gahl WA, Lefrancois S, Morales CR, Pshezhetsky AV. ntracellular distribution of lysosomal sialidase is controlled by the internalization signal in its cytoplasmic tail. *J Biol Chem.* 276(49), 46172-81 (2001).

Mancini GM, A.T. Hoogeveen, H. Galjaard, J.E. Mansson, and L. Svennerholm. Ganglioside GM1 metabolism in living human fibroblasts with beta-galactosidase deficiency *Hum. Genet.* 73(1), 35-38 (1986).

Manzi AE, Higa HH, Diaz S, Varki A. Intramolecular self-cleavage of polysialic acid. *J Biol Chem.* 269(38):23617-24 (1994).

Maroteaux P., Humbel R., Strecker G., Michalski J.C., Mande R.. [A new type of sialidosis with kidney disease: nephrosialidosis. I. Clinical, radiological and nosological study]. *Arch Fr Pediatr.* 35(8), 819-29. (1978) French.

Martin RA, Slaugh R, Natowicz M, Pearlman K, Orvisky E, Krasnewich D, Kleta R, Huizing M, Gahl WA. Sialic acid storage disease of the Salla phenotype in American monozygous twin female sibs. *Am J Med Genet A.* 120(1):23-7 (2003).

Matoth, Y., Chazan, S.; Cnaan, A., Gelernter, I., Klibansky, C. Frequency of carriers of chronic (type I) Gaucher disease in Ashkenazi Jews. *Am. J.Med. Genet.* 27, 561-565 (1987).

Matsuda J, O. Suzuki, A. Oshima, A. Ogura, Y. Noguchi, Y. Yamamoto, T. Asano, K. Takimoto, K. Sukegawa, Y. Suzuki, and M. Naiki. Beta-galactosidase-deficient mouse as an animal model for GM1-gangliosidosis. *Glycoconj. J.* 14(6), 729-736 (1997).

Meikle P.J., Hopwood J.J., Clague A.E., Carey W.F. Prevalence of lysosomal storage disorders. *JAMA.* 281(3), 249-54 (1999).

Milner, C.M., S.V. Smith, M.B. Carrillo, G.L. Taylor, M. Hollinshead, and R.D. Campbell. Identification of a sialidase encoded in the human major histocompatibility complex. *J. Biol. Chem.*, 272, 4549-4558 (1997).

Miyagi T, K. Hata, A. Hasegawa, and T. Aoyagi. Differential effect of various inhibitors on four types of rat sialidase. *Glycoconj J,* 10(1), 45-49 (1993).

Miyagi,T., J. Sagawa, K. Konno, and S. Tsuiki. Immunological discrimination of intralysosomal, cytosolic, and two membrane sialidases present in rat tissues. *J. Biochem.* 107, 794-798 (1990).

Miyagi T, K. Hata, K. Konno, and S. Tsuiki. Multiple forms of mammalian sialidase: altered expression in carcinogenesis. *Tohoku J. Exp. Med.* 168(2), 223-229 (1992).

Miyagi T, K. Hata, A. Hasegawa, and T. Aoyagi. Differential effect of various inhibitors on four types of rat sialidase. *Glycoconj J,* 10(1), 45-49 (1993).

Miyagi T and Tsuiki S. Purification and characterization of cytosolic sialidase from rat liver.*J Biol Chem.* 260(11), 6710-6 (1985).

Miyagi T., Wada T., Iwamatsu A., Hata K., Yoshikawa Y., Tokuyama S., Sawada M. Molecular cloning and characterization of a plasma membrane-associated sialidase specific for gangliosides. *J Biol Chem.* 274(8), 5004-11 (1999).

Monti E., Bassi M.T., Papini N., Riboni M., Manzoni M., Venerando B., Croci G., Preti A., Ballabio A., Tettamanti G., Borsani G. Identification and expression of NEU3, a novel human sialidase associated to the plasma membrane. *Biochem J.* 349(Pt 1), 343-351 (2000).

Monti E, A. Preti, C. Nesti, A. Ballabio, and G. Borsani. Expression of a novel human sialidase encoded by the NEU2 gene. *Glycobiology* 9(12),1313-1321 (1999).

Morreau, H., N.J. Galjart, R. Willemsen, N. Gillemans, X.Y. Zhou, and A. d'Azzo. Human lysosomal protective protein. Glycosylation, intracellular transport,

and association with -galactosidase in the endoplasmic reticulum. *J. Biol. Chem.* 267, 17949-17956 (1992).

Munday J, Floyd H, Crocker PR. Sialic acid binding receptors (siglecs) expressed by macrophages. *J Leukoc Biol.* 66(5):705-11 (1999).

Muerkoster S, Rocha M, Crocker PR, Schirrmacher V, Umansky V. Sialoadhesin-positive host macrophages play an essential role in graft-versus-leukemia reactivity in mice. *Blood.* 93(12):4375-86 (1999).

Naganawa Y, K. Itoh, M. Shimmoto, K. Takiguchi, H. Doi, Y. Nishizawa, T. Kobayashi, S. Kamei, K.E. Lukong, A.V. Pshezhetsky, and H. Sakuraba. Molecular and structural studies of Japanese patients with sialidosis type 1. *J. Hum. Genet.* 45(4), 241-249 (2000).

Nan X, Carubelli I, Stamatos NM. Sialidase expression in activated human T lymphocytes influences production of IFN-{gamma}. *J Leukoc Biol.* 81(1):284-96 (2007).

Naraparaju VR. and N. Yamamoto. Roles of beta-galactosidase of B lymphocytes and sialidase of T lymphocytes in inflammation-primed activation of macrophages *Immunol. Lett.* 43(3), 143-148 (1994).

Nishiyama Y., Sakamoto S., Katayama Y., Mishina M., Terashi A. [Neuroradiological findings on cerebral blood flow and metabolism of a case of adult onset sialidosis]. *Rinsho Shinkeigaku.* 37(11), 982-6 (1997) Japanese.

Neufeld E.F. Lysosomal storage diseases. *Annu Rev Biochem.* 60:257-80 (1991).

O'Brien, J.S. The cherry red spot-myoclonus syndrome: a newly recognized inherited lysosomal storage disease due to acid neuraminidase deficiency. *Clin. Genet.,* 14, 55-60 (1979).

O'Brien JS, M.B. Stern, B.H. Landing, J.K. O'Brien, and G.N. Donnell. Generalized gangliosidosis. Another inborn error of metabolism? *Am. J. Dis. Child.* 109, 338 (1965).

O'Brien JS, E. Gugler, A. Giedion, U. Wiessmann, N. Herschkowitz, C. Meier, and J. Leroy. Spondyloepiphyseal dysplasia, corneal clouding, normal intelligence and acid beta-galactosidase deficiency. *Clin Genet* 9(5), 495-504 (1976).

O'Brien JS, Warner TG. Sialidosis: delineation of subtypes by neuraminidase assay. *Clin Genet.* 17(1), 35-8 (1980).

Olcay L., Gumruk F., Boduroglu K., Coskun T., Tuncbilek E. : Anaemia and thrombocytopenia due to haemophagocytosis in a 7-month-old boy with galactosialidosis. *J. Inherit. Metab. Dis.* 21, 679-680 (1998).

Oohira, T., N. Nagata, I. Akaboshi, I. Matsuda, and S. Naito. The infantile form of sialidosis type II associated with congenital adrenal hyperplasia: possible linkage between HLA and the neuraminidase deficiency gene. *Hum. Genet.* 70, 341-343 (1985).

Orsel J.G., Sincock P.M., Krise J.P., Pfeffer S.R. Recognition of the 300-kDa mannose 6-phosphate receptor cytoplasmic domain by 47-kDa tail-interacting protein. *Proc Natl Acad Sci U S A.* 97(16), 9047-51 (2000).

Pappu BP, Shrikant PA. Alteration of cell surface sialylation regulates antigen-induced naive CD8+ T cell responses. *J Immunol.* 173(1):275-84 (2004).

Patel MS, Callahan JW, Zhang S, Chan AK, Unger S, Levin AV, SkomorowskiMA, Feigenbaum AS, O'Brien K, Hellmann J, Ryan G, Velsher L, Chitayat D. Early-infantile galactosialidosis: prenatal presentation and postnatal follow-up. *Am J Med Genet.* 85(1), 38-47 (1999).

Pearse BM, Smith CJ, Owen DJ. Clathrin coat construction in endocytosis. *Curr Opin Struct Biol.* 10(2), 220-8 (2000).

Peters C. and K. von Figura. Biogenesis of lysosomal membranes. *FEBS Lett* 346(1), 108-114 (1994).

Petersen, G. M., Rotter, J. I., Cantor, R. M., Field, L. L., Greenwald, S., Lim, J. S. T., Roy, C., Schoenfeld, V., Lowden, J. A., Kaback, M. M. The Tay-Sachs disease gene in North American Jewish populations: geographicvariations and origin. *Am. J. Hum. Genet.* 35, 1258-1269 (1983).

Pinsky L, Miller J, Shanfield B, Watters G, Wolfe L.S. G_{M1} gangliosidosis in skin fibroblast culture: enzymatic differences between types 1 and 2 and observations on a third variant. *Am J Hum Genet.* 26(5), 563-77 (1974).

Poorthuis B.J., Wevers R.A., Kleijer W.J., Groener J.E., de Jong J.G., van Weely S, Niezen-Konin KE, van Diggelen OP. The frequency of lysosomal storage diseases in The Netherlands. *Hum Genet.* 105(1-2), 151-6 (1999).

Potier M, Michaud L, Tranchemontagne J, Thauvette L. Structure of the lysosomal neuraminidase-β-galactosidase-carboxypeptidase multienzyme complex. *Biochem. J.* 267,197-202 (1990).

Provenzale JM, Barboriak DP, Sims K. Neuroradiologic findings in fucosidosis, a rare lysosomal storage disease. *AJNR Am J Neuroradiol.* 16(4 Suppl), 809-13 (1995).

Pshezhetsky AV, Ashmarina M. Lysosomal multienzyme complex: biochemistry, genetics, and molecular pathophysiology. *Prog Nucleic Acid Res Mol Biol.* 69:81-11 (2001).

Pshezhetsky, A.V. and M. Potier. Association of N-acetyl-galactosamine-6-sulfate sulfatase with the multienzyme lysosomal complex of -galactosidase, cathepsin A and neuraminidase: possible implication for intralysosomal catabolism of keratan sulfate. *J. Biol. Chem.* 271, 28359-28365 (1996).

Pshezhetsky, A.V. and M. Potier. Direct affinity purification and supramolecular organization of human lysosomal cathepsin A. *Arch. Biochem. and Biophys.* 313, 64-70 (1994).

Pshezhetsky, A.V., Richard, C., Michaud, L., Igdoura, S., Wang, S., Elsliger, M. A., Qu, J., Leclerc, D., Gravel, R., Dallaire, L. and Potier, M. Cloning, expression and chromosomal mapping of human lysosomal sialidase and characterization of mutations in sialidosis. *Nat. Genet.*, 15, 316-20 (1997).

Raas-Rothschild A, Cormier-Daire V, Bao M, Genin E, Salomon R, Brewer K, Zeigler M, Mandel H, Toth S, Roe B, Munnich A, Canfield WM. Molecular

basis of variant pseudo-hurler polydystrophy. *J Clin Invest.* 105(5), 673-81 (2000).

Ransone LJ, Verma IM. Nuclear proto-oncogenes fos and jun. *Annu Rev Cell Biol.* 6:539-57 (1990).

Rapin, I., S. Goldfischer, R. Katzman, J. Engel Jr, and J.S. O'Brien. The cherry-red spot-myoclonus syndrome. *Ann. Neurol.,* 3, 234-242 (1978).

Rauscher FJ 3rd, Cohen DR, Curran T, Bos TJ, Vogt PK, Bohmann D, Tjian R, Franza BR Jr. Fos-associated protein p39 is the product of the jun proto-oncogene. *Science.* 240(4855):1010-6 (1988).

Remington S.J. Serine carboxypeptidases: a new and versatile family of enzymes. *Curr Opin Biotechnol.* 4(4), 462-8 (1993).

Richard C, J. Tranchemontagne, M.A. Elsliger, G.A. Mitchell, M. Potier, and A.V. Pshezhetsky. Molecular pathology of galactosialidosis in a patient affected with two new frameshift mutations in the cathepsin A/protective protein gene. *Hum. Mutat.* 11(6), 461-469 (1998).

Robbins A.R., Myerowitz R., Youle R.., Murray G.J., Neville D.M. Jr. The mannose 6-phosphate receptor of Chinese Hamster ovary cells. Isolation of mutants with altered receptors. *J Biol Chem.* 256(20), 10618-22 (1981).

Roggentin T, Kleineidam RG, Majewski DM, Tirpitz D, Roggentin P, Schauer R. An immunoassay for the rapid and specific detection of three sialidase-producing clostridia causing gas gangrene. *J Immunol Methods.* 157(1-2), 125-33 (1993).

Rottier, R.J. E. Bonten, and A. d'Azzo A point mutation in the neu-1 locus causes the neuraminidase defect in the SM/J mouse. *Hum. Mol. Genetics,* 7, 313-321 (1998).

Rudenko G, E. Bonten, W.G. Hol, and A. d'Azzo. The atomic model of the human protective protein/cathepsin A suggests a structural basis for galactosialidosis. *Proc. Natl Acad. Sci. U S A* 95(2), 621-625 (1998).

Rudenko, G., E. Bonten, A. d'Azzo, and W.G.J. Hol. Three-dimensional structure of the human protective protein: structure of the precursor form suggests a complex activation mechanism. *Structure* 3, 1249-1259 (1995).

Sahagian G.G. The mannose 6-phosphate receptor: function, biosynthesis and translocation. *Biol Cell.* 51(2), 207-14 (1984).

Sakuraba H, Y. Suzuki, M. Akagi, M. Sakai, and N. Amano. beta-Galactosidase-neuraminidase deficiency (galactosialidosis): clinical, pathological, and enzymatic studies in a postmortem case. *Ann. Neurol.* 13(5), 497-503 (1983).

Sallusto F, Lanzavecchia A. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J Exp Med.* 179(4):1109-18 (1994).

Sato K and Miyagi T. Genomic organization and the 5'-upstream sequence of the rat cytosolic sialidase gene. *Glycobiology.* 5(5), 511-6 (1995).

Sato K, Miyagi T. Involvement of an endogenous sialidase in skeletal muscle cell differentiation. *Biochem Biophys Res Commun.* 221(3), 826-30 (1996)

Schauer T., Trends Biochem. *Sci.* 10, 357-360 (1985).

Schenkman S, Chaves L.B., Pontes de Carvalho L.C., Eichinger D. A proteolytic fragmentof Trypanosoma cruzi trans-sialidase lacking the carboxyl-terminal domain is active, monomeric, and generates antibodies that inhibit enzymatic activity.*J Biol Chem.* 269(11), 7970-5 (1994).

Schneider-Jakob, H.R. and M. Cantz. Lysosomal and plasma membrane ganglioside GM3 sialidases of cultured human fibroblasts. Differentiation by detergents and inhibitors. *Biol. Chem. Hoppe-Seyler* 372, 443-450 (1991).

Schonthal A, Herrlich P, Rahmsdorf HJ, Ponta H. Requirement for fos gene expression in the transcriptional activation of collagenase by other oncogenes and phorbol esters. *Cell.* 54(3):325-34(1988).

Schweizer A, Stahl P.D. and Rohrer J. A Di-aromatic motif in the cytosolic tail of the mannose receptor mediates endosomal sorting. *J Biol Chem.* 275(38), 29694-700 (2000).

Seyrantepe V, Landry K, Trudel S, Hassan JA, Morales CR, Pshezhetsky AV. Neu4, a novel human lysosomal lumen sialidase, confers normal phenotype to sialidosis and galactosialidosis cells. *J Biol Chem.* 279(35), 37021-9 (2004).

Shen W, Ladisch S. Ganglioside GD1a impedes lipopolysaccharide-induced maturation of human dendritic cells. Cell Immunol. 220(2):125-33 (2002).

Shimmoto, M., Y. Nakahori, I. Matsuhita, T. Shinka, Y. Kuroki, K. Itoh, and H. Sakuraba. A human protective protein gene partially overlaps the gene encoding phospholipid transfer protein on the complementary strand of DNA. *Biochem. Biphys. Res. Commun.* 220, 802-806 (1996).

Soggs, R.L., L. Steinman, B. Rathjen, B.R. Tharp, J.S. O'Brien and K.R. Kenyon Cherry-rep spot-myoclonus syndrome. *Ophtalmology,* 86, 1861-1874 (1979).

Stamatos NM, Curreli S, Zella D, Cross AS. Desialylation of glycoconjugates on the surface of monocytes activates the extracellular signal-related kinases ERK 1/2 and results in enhanced production of specific cytokines. *J Leukoc Biol.* 75(2):307-13 (2004).

Stone DL, and Sidransky E. Hydrops fetalis: lysosomal storage disorders in extremis. *Adv Pediatr.* 46, 409-40 (1999).

Strecker G, Herlant-Peers MC, Fournet B, Montreul J. Structure of seven oligosaccharides excreted in the urine of a patient with Sandhoff's disease (G_{M2} gangliosidosis-variant O). *Eur J Biochem.* 81(1), 165-71 (1977).

Suzuki Y., Nanba E., Tsuji A., Yang R.C., Okamura-Oho Y., Yamanaka T. Clinical and genetic heterogeneity in galactosialidosis. *Brain Dysfunction* 1, 285-293 (1988) .

Takano T., Shimmoto M., Fukuara Y., Itoh K., Kase R., Takiyama N., Kobayashi T., Oshima A., Sakuraba H., Suzuki Y. Galactosialidosis: Clinical and molecular analysis of 19 Japanese patients. *Brain Dysfunction* 4, 271-280 (1991).

Tang H.B., DiMango E., Bryan R., Gambello M., Iglewski B.H., Goldberg J.B., Prince A. Contribution of specific Pseudomonas aeruginosa virulence factors to pathogenesisof pneumonia in a neonatal mouse model of infection. *Infect Immun.* 64(1), 37-43 (1996).

Tanaka S, Kiuchi Y, Numazawa S, Oguchi K, Yoshida T, Kuroiwa Y. Changes in glutamate receptors, c-fos mRNA expression and activator protein-1 (AP-1) DNA binding activity in the brain of phenobarbital-dependent and -withdrawn rats. *Brain Res.* 756(1-2):35-45 (1997).

Thomas G. H. ,Tipton, R. E., Ch'ien L. T., Reynolds L. W., Miller C. S. : Sialidase (alpha-N-acetyl neuraminidase) deficiency: the enzyme defect in an adultwith macular cherry red spots and myoclonus without dementia. *Clin. Genet.* 13,369-379 (1978).

Tokuyama S, S. Moriya, S. Taniguchi, A. Yasui, J. Miyazaki, S. Orikasa, and T. Miyagi. Suppression of pulmonary metastasis in murine B16 melanoma cells by transfection of a sialidase cDNA. *Int. J. Cancer* 73(3), 410-415 (1997).

Tranchemontagne J, Michaud L, Potier M. Deficient lysosomal carboxypeptidase activity in galactosialidosis. *Biochem Biophys Res Commun.* 168(1):22-9 (1990).

Tsuchiya S, Yamabe M, Yamaguchi Y, Kobayashi Y, Konno T, Tada K. Establishment and characterization of a human acute monocytic leukemia cell line (THP-1). *Int J Cancer.* 26(2):171-6 (1980).

Turley SJ, Inaba K, Garrett WS, Ebersold M, Unternaehrer J, Steinman RM, Mellman I. Transport of peptide-MHC class II complexes in developing dendritic cells. *Science.* 288(5465):522-7 (2000).

Tylki-Szymanska A., Lugowska A., Czartoryska B. Neuraminidase deficiency presenting as a nephrosialidosis: the first case detected in Poland. *Acta Paediatr Jpn.* 38(5), 529-32m (1996).

Ulrich-Bott B, B. Klem, R. Kaiser, J. Spranger, and M. Cantz. Lysosomal sialidase deficiency: increased ganglioside content in autopsy tissues of a sialidosis patient. *Enzyme* 38(1-4), 262-266 (1987).

van den Berg TK, Breve JJ, Damoiseaux JG, Dopp EA, Kelm S, Crocker PR, Dijkstra CD, Kraal G. Sialoadhesin on macrophages: its identification as a lymphocyte adhesion molecule. *J Exp Med.* 176(3):647-55 (1992).

van der Horst GT, Galjart NJ, d'Azzo A, Galjaard H, Verheijen FW. Identification and in vitro reconstitution of lysosomal neuraminidase from human placenta. *J Biol Chem.* 264(2),1317-22 (1989).

van der Spoel, A., E. Bonten, and A. Azzo. Transport of human lysosomal neuraminidase to mature lysosomes requires protective protein/cathepsin A. *EMBO J.*, 17, 1588-1597 (1998).

van Diggelen OP, A.T. Hoogeveen, P.J. Smith, A.J. Reuser, and H. Galjaard. Enhanced proteolytic degradation of normal beta-galactosidase in the lysosomal storage disease with combined beta-galactosidase and neuraminidase deficiency. *Biochim. Biophys. Acta* 703(1), 69-76 (1982).

Van Hoof, F [Lysosomal Diseases], [French], Bruxelles. *Medical*, 56, 193-203 (1976).

Van Pelt J., Van Kuik J.A., Kamerling J.P., Vliegenthart J.F., Van Diggelen O.P., Galjaard H. Storage of sialic acid-containing carbohydrates in the placenta of a human galactosialidosis fetus. Isolation and structural characterization of 16 sialyloligosaccharides. *Eur J Biochem.* 177(2), 327-38 (1988).

Verheijen FW, Janse HC, van Diggelen OP, Bakker HD, Loonen MC, Durand P, Galjaard H Two genetically different mu-nana neuraminidases in human leucocytes. *Biochem. Biophys. Res. Commun.,* 117, 470-478 (1983).

Verheijen FW, Palmeri S, Hoogeveen AT, Galjaard H. Human placental neuraminidase. Activation, stabilization and association with beta-galactosidase and its protective protein. *Eur J Biochem.*149(2), 315-21 (1985).

Vinogradova MV, L. Michaud, A.V. Mezentsev, K.E. Lukong, M. El-Alfy, C.R. Morales, M. Potier, and A.V. Pshezhetsky. Molecular mechanism of lysosomal sialidase deficiency in galactosialidosis involves its rapid degradation. *Biochem. J.* 330 (Pt 2), 641-650 (1998).

Wang P, Zhang J, Bian H, Wu P, Kuvelkar R, Kung TT, Crawley Y, Egan RW, Billah MM. Induction of lysosomal and plasma membrane-bound sialidases in human T-cells via T-cell receptor. *Biochem J.* 380(Pt 2):425-33 (2004).

Warner T.G., Chang J., Ferrari J., Harris R., McNerney T., Bennett G., Burnier J., Sliwkowski M.B. Isolation and properties of a soluble sialidase from the culture fluid of Chinese hamster ovary cells. *Glycobiology.* 3(5), 455-63 (1993).

Wenger D.A., Tarby T.J., Wharton C. Macular cherry-red spots and myoclonus with dementia: coexistent neuraminidase and beta-galactosidase deficiencies. *Biochem Biophys Res Commun.* 82(2), 589-95 (1978).

Yoshino H, K. Miyashita, N. Miyatani, T. Ariga, Y. Hashimoto, S. Tsuji, K. Oyanagi, E. Ohama, F. Ikuta, A. Suzuki, et al. Abnormal glycosphingolipid metabolism in the nervous system of galactosialidosis. *J. Neurol. Sci.* 97(1), 53-65 (1990).

Zhang S. and Callahan J.W. (1994) The biochemistry and clinical features of galactosialidosis. *Biochim. Biophys Acta* 1225,244-254. (1996).

Zhou, X.Y., H. Morreau, R. Rottier, D. Davis, E. Bonten, N. Gillemans, D. Wenger, F.G. Grosveld, P. Doherty, K. Suzuki, G. Grosveld, and A. d'Azzo. Mouse model for the lysosomal disorder galactosialidosis and correction of the phenotype with overexpressing erythroid precursor cells. *Genes & Dev.* 9, 2623-2634 (1995).

Zhou X.Y., van der Spoel A., Rottier R., Hale G., Willemsen R., Berry G.T., Strisciuglio P.,Morrone A.Zammarchi E. Andria G., d'Azzo A. Molecular and biochemical analysis of protective protein/cathepsin A mutations: correlation with clinical severity in galactosialidosis. *Hum Mol Genet.* 5(12), 1977-87 (1996).