Study of Human-Specific Microglial Receptor Siglec-11 and Generation of Transgenic Mice Expressing Human Siglec-11

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Dedicated to my families

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ABBREVIATIONS

AAV	adeno-associated virus
Ab	antibody
AD	Alzheimer's disease
ALS	amyotrophic lateral sclerosis
ANOVA	analysis of variance
APP	amyloid precursor protein
ATP	adenosine triphosphate
Aβ	amyloid β
BBB	blood brain barrier
bFGF	basic fibroblast growth factor
BM	bone marrow
BMSC	bone marrow stem cells
bp	base pairs
BSA	bovine serum albumine
CCR	chemokine receptor
CD	cluster of differentiation
CMV	cytomegalovirus
CNS	central nervous system
COX-2	cyclooxygenase-2
cPPT	the central polypurine tract
CRD	carbohydrate-recognition domain
CX3CR-1	fractalkine receptor
DAP12	DNAX activating protein of 12 kD
DAPI	4',6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
dpc	days post-coitum
EAE	experimental autoimmune encephalomyelitis
EB	embryoid bodies
EGF	epidermal growth factor
ERK	extracellular signal-regulated protein kinase
ES	Embryonic Stem
FACS	fluorescence activated cell sorting
FBS	fetal bovine serum
FITC	fluoro-isothiocyanate
FIV	feline immunodeficiency virus
FSH	follicle-stimulating hormone
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFP	green fluorescence protein
GM-CSF	granulocyte-macrophage colony stimulating factor
GRB2	growth-factor-receptor-bound protein 2
h	hours

hCG	human Chorionic Gonadotropin
HEK	human embryonic kidney
HIV	human immunodeficiency virus
HRP	horseradish peroxidase
Iba1	ionized calcium-binding adapter molecule 1
ICAM	intercellular adhesion molecules
ICM	inner cell mass
INF	Interferon
Ig	immunoglobulin
IgSF	immunoglobulin superfamily
IL	Interleukin
ITAM	immunoreceptor tyrosine based activation motif
ITIM	immunoreceptor tyrosine based inhibition motif
iNOS	inducible NOS
ITSFn	insulin transferring selenit fibronectin
kg	kilogram
L	liter
LBP	Lipopolysacharide binding protein
LFA	leukocyte function-associated molecule
LH	luteinizing hormone
LIF	leukemia inhibitory factor
LPS	lipopolysaccharides
LTRs	long terminal repeats
M	molar
mAb	monocolonal antibody
MAG	myelin-associated glycoprotein
MAPK	mitogen-activated protein kinase
MCP	monocyte chemoattractant protein
MEF	mouse embryonic fibroblast
mES	murine embryonic stem
mg	miligram
MHC	major histocompatibility complex
min	minutes
ml	milliliter
mm	millimeter
mM	millimolar
MS	Multiple Sclerosis
NCAM	Neural Cell Adhesion Molecule
Neu5,(7)9Ac2	5,(7)9-N,O-diacetylneuraminic acid
Neu5Ac	N-acetylneuraminic acid
Neu5Gc	N-glycolylneuraminic acid
ng	nanogram
NF-κB	nuclear factor-kappa B
nGS	normal goat serum

nGOS	neuronal NOS
NK	natural killer
NO	nitric oxide
NOS	nitric oxide synthase
PAR-1	protease-activated receptor-1
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerytin
PFA	paraformaldehyde
PGK	phosphoglycerate kinase
PI3-K	Phosphoinositide 3-kinases
PKB	protein kinase B
PLL	poly-L-lysine
PMS	Pregnant Mare's Serum
PSA	Poly sialic acid
PTP	protein tyrosine phosphatase
RAGE	receptors for advanced glycosylated endproducts
RNA	ribonucleic acid
RRE	the Rev response element
RT	room temperature
RT-PCR	reverse transcription PCR
SEM	standard error of the mean
SHIP	SH2-domain-containing inositol polyphhosphate 5-pohsphatase
SHP	SH2 domain-containing phosphatases
Siglec	Sia-recognizing Ig-superfamily lectins
SIN	self-inactivating
Sn	Sialoadhesin
SSEA	specific cell surface antigen
SMP	Schwann cell myelin protein
syk	spleen tyrosine kinase
TAE	Tris-acetate
TBE	Tris-borate
TGF	transforming growth factor
TLR	toll-like receptors
TNF	tumor necrosis factor
TPA	12-O-tetradecanoylphorbol-13-acetate
TU	Transducing Units
UDP	uridine triphosphate
VSV-G	G protein of the vescular stomatitis virus
WPRE	wood chuck hepatitis virus post-transcriptional regulatory element
µl	microliter
µm	micrometer
µg	microgram
Ψ	retroviral packaging signal

1 INTRODUCTION

1.1 Siglecs

1.1.1 Definition and nomenclature of Siglecs

Animal glycan-recognizing proteins can be broadly classified into two groups: lectins, which typically contain an evolutionarily conserved carbohydrate-recognition domain (CRD), and sulfated glycosaminoglycan (SGAG)-binding proteins, which appear to have been evolved by convergent evolution. Proteins other than antibodies and T-cell receptors that mediate glycan recognition via immunoglobulin (Ig)-like domains are called "I-type lectins". The major homologous subfamily of I-type lectins with sialic acid (Sia)-binding properties and characteristic amino-terminal structural features are called the "Siglecs" (Sia-recognizing Ig-superfamily lectins).

Criteria for the inclusion of the immunoglobulin superfamily-related proteins as Siglecs were defined as: (1) the ability to recognize sialylated glycans mediated by the N-terminal V-set domain via well-characterized molecular interactions, including a key arginine (Arg) residue that forms a salt bridge with the carboxylate group of sialic acid, and (2) significant sequence similarity within the N-terminal V-set and adjoining C2-set domains.

There are currently 14 human and 9 mouse molecules that fulfill these criteria (Crocker, Paulson et al. 2007; Cao, Lakner et al. 2008) (Figure 1-1). Scientists in the field established the Siglec nomenclature of naming the members in order of discovery. Thus sialoadhesin (Sn) was given the designation Siglec-1, because it was the first member characterized as a Sia-binding lectin. Furthermore, categorizing CD22 as Siglec-2 and CD33 as Siglec-3, respectively, was useful as a "memory aid". Mammalian myelin-associated glycoprotein (MAG) and avian Schwann cell myelin protein (SMP) were grouped together as Siglec-4a and -4b, respectively, because they are structurally and functionally related. Complexity in nomenclature arises from the fact that orthologs of some Siglecs in certain species have undergone mutations in an "essential" Arg residue required for optimal Sia binding and therefore no longer fulfill all the criteria to be called Siglecs. The first of these was found in

humans and initially called Siglec-L1 (Siglec-like molecule-1) (Angata, Varki et al. 2001). This molecule has a Sia-binding ("essential Arg"-containing) ortholog in the chimpanzee, designated as chimpanzee Siglec-12 (cSiglec-12). The international nomenclature group thus agreed to change the name of hSiglec-L1 to hSiglec-XII (the Roman numeral indicates that it is the Arg-mutated ortholog of cSiglec-12) (Angata 2004). Likewise, the Arg-mutated ortholog of hSiglec-5 in the chimpanzee is designated cSiglec-V, and the Arg-mutated Siglec-6 ortholog in baboon is bSiglec-VI. A primate molecule deleted in humans was discovered by sequencing the chimpanzee Siglec gene cluster and designated as Siglec-13 (Angata, Margulies et al. 2004). In case of rodent CD33/Siglec-3-related Siglecs, alphabetical designations were applied, because it was difficult to assign the human orthologues of all rodents CD33/Siglec-3-related Siglecs.

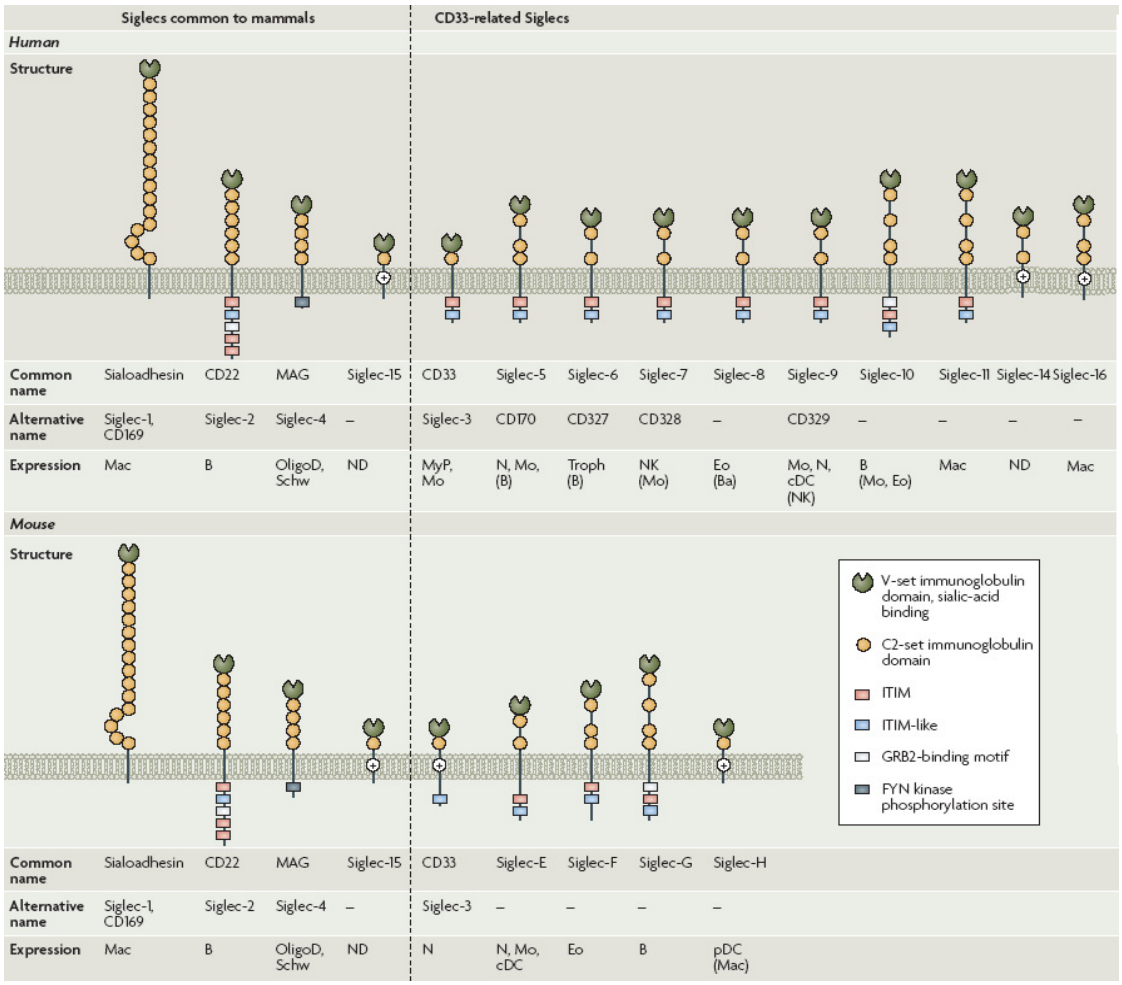


Figure 1-1. Siglec-family proteins in humans and rodents. The brackets indicate low

levels of expression. Siglec-12 in humans has lost the ability to bind sialic acids and is, hence, designated as Siglec-XII (not shown). Abbreviations: B, B cells; Ba, basophils; cDCs, conventional dendritic cells; Eo, eosinophils; GRB2, growth-factor-receptor-bound protein 2; ITIM, immunoreceptor tyrosine-based inhibitory motif; Mac, macrophages; Mo, monocytes; MyP, myeloid progenitors; N, neutrophils; ND, not determined; NK, natural killer cells; OligoD, oligodendrocytes; pDCs, plasmacytoid dendritic cells; Schw, Schwann cells; Troph, trophoblasts. Figure modified from (Paul R. Crocker, 2007).

1.1.2 Subfamilies of Siglecs

On the basis of their sequence similarity and evolutionary conservation, Siglecs can be broadly divided into two groups: an evolutionary conserved subgroup, which includes Siglec-1, -2, -4 and -15, and a CD33/Siglec-3-related subgroup, which appears to be rapidly evolved (Table 1-1). The members of the first group are quite distantly related (~25–30% sequence identity), and have clear orthologues in all mammalian species examined. In comparison, the CD33-related Siglecs share ~50–99% identity but seem to be evolved rapidly by multiple processes, including gene duplication, exon shuffling, exon loss and gene conversion. This has resulted in important differences in the repertoires of CD33-related Siglecs among mammalian species. In humans, there are ten CD33-related Siglecs and one Siglec-like protein, including the recently defined Siglec-16 which was recognized as a pseudogene in the past (Cao, Lakner et al. 2008), whereas in mice there are five CD33-related Siglecs (Siglec-3 and E–H).

Table 1-1. Evolutionary comparison of the two major subgroups of Siglecs. (Vaki et al. 2006)

	Sn/CD22/MAG group	CD33rSiglecs
Orthologs	Single clear-cut orthologs in multiple species	Clear-cut orthologs difficult to define between primates and rodents
Binding specificity	Highly conserved (with minor variations)	Poorly conserved (rapidly evolving)
Cell-type of expression	Highly conserved	Highly variable expression in cells of innate immunity (granulocytes, monocytes, and macrophages)
	CD22 = B cells	
	MAG = glial cells	
	Sn = macrophages	
Domain structure	Highly conserved	Poorly conserved
Evidence for hybrid genes and gene conversion events	No	Yes
Cytosolic tyrosine-based motifs	ITIM only in CD22. None in Sn and MAG (MAG has Tyr-based motif similar to CD33rSiglecs' "distal motif")	Conserved ITIM and ITIM-like motif in most members

CD33rSiglecs, CD33/Siglec-3-related Siglecs; ITIM, immunoreceptor tyrosine-based inhibitory motif; MAG, myelin-associated glycoprotein; Sn, sialoadhesin.

1.1.3 Expression pattern of human Siglecs

Each human Siglec is expressed in a cell type-specific fashion, suggesting involvement in discrete functions. The selective expression of Sn/Siglec-1, CD22/Siglec-2, and MAG/Siglec-4 on tissue macrophages, mature B cells, and glial cells, respectively, appears to be conserved amongst all mammalian species studied so far.

The CD33-related Siglecs appear to be variably distributed amongst cell types in the immune system, with significant overlaps (Figure 1-2). The striking exception are T cells in which very low expression of Siglecs is seen (Razi and Varki 1998), primarily Siglec-7 and -9 on a subset of CD8+ T cells in some humans (Nicoll, Ni et al. 1999; Zhang, Nicoll et al. 2000; Ikehara, Ikehara et al. 2004). Also, Siglec-6 is expressed in placental trophoblast cells (Patel, Brinkman-Van der Linden et al. 1999).

The cell type-specificity of human and mouse CD33-related Siglecs often do not follow their presumed orthologous relationships, for example, although human CD33/ Siglec-3 is highly expressed on mature monocytes, mouse CD33/Siglec-3 is expressed only on granulocytes (Brinkman-Van der Linden, Angata et al. 2003). Most CD33-related Siglecs are found on multiple leukocyte types to varying extents, for example, human CD33/Siglec-3, -5, -7, -9, and -10 are expressed on circulating monocytes. When monocytes are differentiated into macrophages or stimulated with lipopolysaccharide (LPS), they retain the expression of these Siglecs (Lock, Zhang et al. 2004). In comparison, monocyte-derived dendritic cells down-modulate Siglec-7 and -9 following maturation with LPS, and plasmacytoid dendritic cells in human blood express only Siglec-5. In a few instances, certain CD33-related Siglecs show expression predominantly restricted to one cell type. Although human Siglec-7 is found at low levels on granulocytes and monocytes, relatively high levels are found on a major subset of NK cells and a minor subset of CD8+ T cells (Nicoll, Ni et al. 1999). Siglec-8 could be detected only on eosinophils (Floyd, Ni et al. 2000).

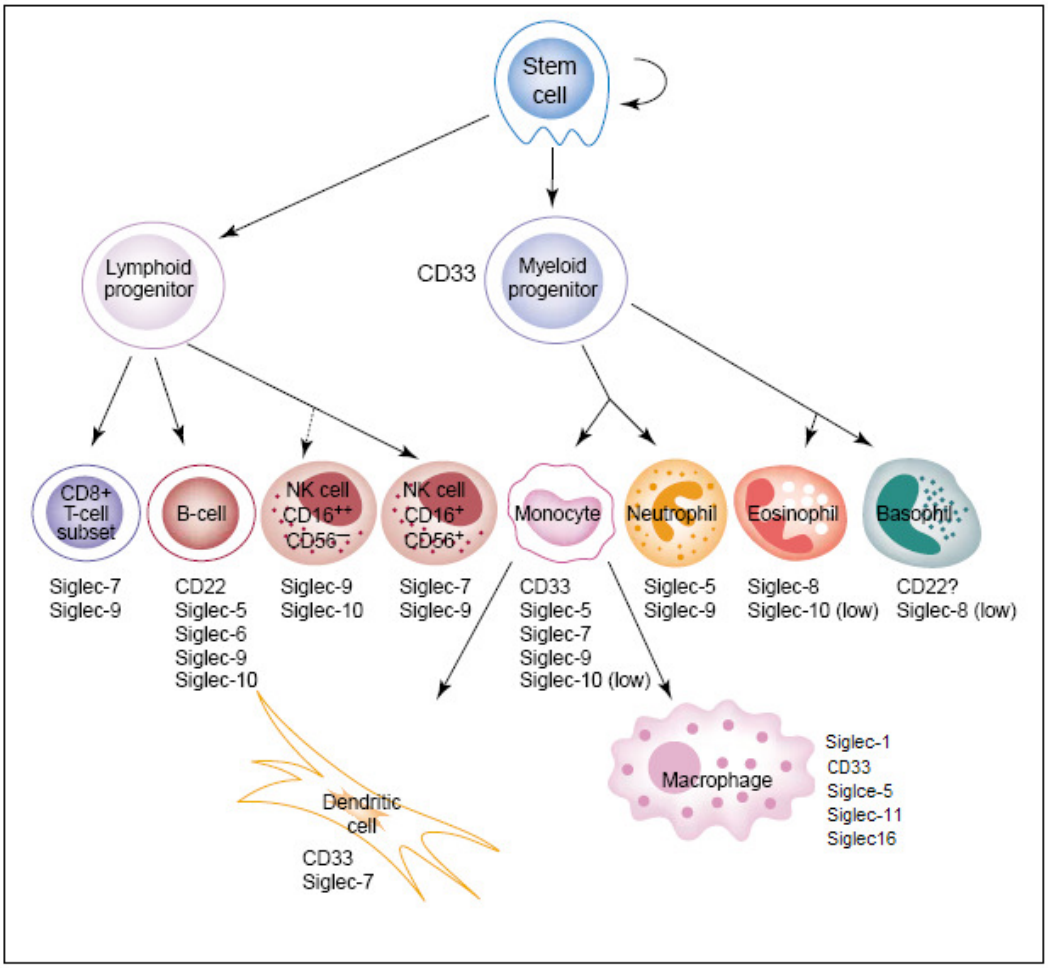


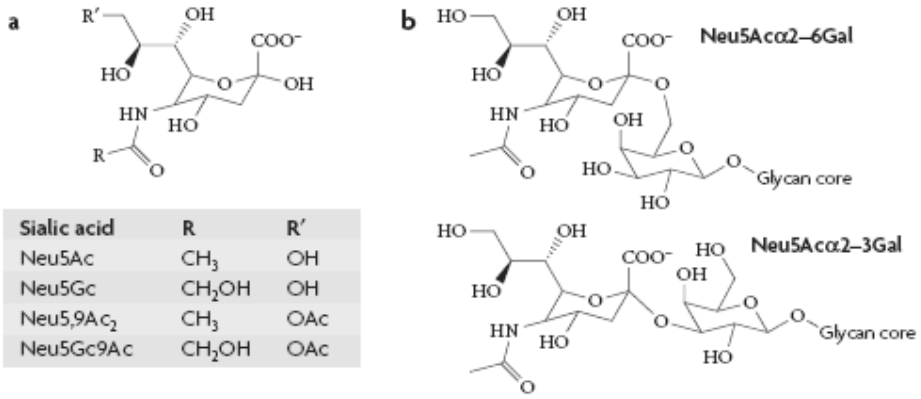
Figure 1-2. Expression pattern of human Siglecs within the hematopoietic system. Abbreviation: NK, natural killer. Figure modified from (Crocker and Varki 2001).

1.1.4 Ligands of Siglecs

1.1.4.1 Sialic acid

Sialic acid (Sia) refers to a family of sugars that are typically found at the outermost end of glycan chains of all cell types (Schauer 2000; Angata and Varki 2002; Varki 2007). These acidic sugars with a nine-carbon backbone are mostly derived from N-acetylneuraminic acid (Neu5Ac). Although there are more than 50 forms of naturally occurring sialic acid, mammals mainly express Neu5Ac, N-glycolylneuraminic acid (Neu5Gc) and 5, (7)9-N, O-diacetylneuraminic acid (Neu5,(7)9Ac2) (Figure 1-3). Humans lack Neu5Gc owing to a mutation in the CMAH (cytidine monophosphate-N-acetylneuraminic acid hydroxylase)

gene, which encodes the enzyme required for the conversion of Neu5Ac to Neu5Gc. Sialic acids are usually located at the exposed, non-reducing ends of oligosaccharide chains, and are transferred using α2-3, α2-6 or α2-8 linkages to subterminal sugars by a family of about 20 sialyltransferases.



Sialic acid	R	R′
Neu5Ac	CH_3	OH
Neu5Gc	CH_2OH	OH
Neu5,9Ac_2	CH_3	OAc
Neu5Gc9Ac	CH_2OH	OAc

Figure 1-3. Sialic acids. a. Sketch of sialic acid with a nine-carbon backbone. In mammals sialic acid is commonly modified at the R and R′ positions with the substituents indicated. b. Two common sialoside sequences recognized as low-affinity ligands by many Siglecs are shown. Figure modified from (Crocker, Paulson et al. 2007).

Sialic acids decorate all cell surfaces and most secreted proteins of vertebrates and 'higher' invertebrates, mediating or modulating a variety of normal and pathological processes. First, by virtue of their negative charge and hydrophilicity, sialic acids have many structural and modulatory roles. In a second category of functions, sialic acids serve as components of binding sites for various pathogens and toxins (Schauer 2000; Lehmann, Tiralongo et al. 2006), such as human influenza A and C, or Helicobacter pylori. In most such interactions, a pathogen binding protein recognizes certain forms of sialic acids presented in specific linkages to a defined underlying sugar chain. Although this recognition is detrimental to the host expressing the cognate sialic acids, these molecules have nevertheless persisted on all cell types in all vertebrates for a long evolutionary time. Thus, a third set of function is the interaction with sialic acid binding proteins, which are intrinsic to the organisms. A final class of functions is "molecular mimicry", in which successful microbial pathogens decorate themselves with sialic acids, which assist in evasion of host immunity (Vimr, Kalivoda et al. 2004).

1.1.4.2 Structural feature of Siglecs for Sia-binding

Most of the functional Siglecs studied to date have a conserved Arg residue in the V set domain that is required for optimal recognition of sialic acids. All Siglecs (other than Siglec-XII) contain an odd number (typically 3) of cysteine residues in the first and second Ig-like domains. Several other amino acid residues have also been defined to have direct contacts with sialylated ligands. For example, Trp2 and Trp106 in Sn/Siglec-1, and tyrosine 26 (Tyr26) and tryptophan 132 (Trp132) in Siglec-7 (Yamaji, Teranishi et al. 2002) are reported to be involved in direct contacts with sialylated ligands. However, these features are not always common to the other siglecs.

1.1.4.3 Recognition of Sias and their linkages by Siglecs

In general, Siglecs show low affinity (a K_d of 0.1-3 mM) for the sialic acid Neu5Ac α2-3 and α2-6 linkages to galactose ((Neu5Ac(α2-3)Gal and Neu5Ac(α2-6)Gal) that are commonly found as terminal sequences on glycans of glycoproteins and glycolipids of most mammalian cells (Bakker, Piperi et al. 2002; Blixt, Collins et al. 2003). And Siglecs have an overlapping specificity for such sialosides (sialic acid-containing glycans). However, when examined for their ability to recognize a diverse set of natural sialoside structures found in mammalian species, each Siglec shows a characteristic specificity profile for the types of sialic acid (Neu5Ac or Neu5Gc) and also for the types of linkage to subterminal sugars. For example, CD22 is unique in having a strong preference for Neu5Ac (α2-6) Gal and Neu5Gc(α2-6)Gal structures (Powell, Sgroi et al. 1993; Kelm, Schauer et al. 1994). Siglec-7 and Siglec-11 prefer sialosides with the Neu5Ac (α2-8) Neu5Ac structure (Yamaji, Teranishi et al. 2002; Hayakawa, Angata et al. 2005). Of particular interest is the evolutionary loss of Neu5Gc in humans, as Neu5Gc is the preferred ligand for at least some Siglecs in the closely related great apes (Sonnenburg, Altheide et al. 2004).

Furthermore, other aspects of the Sia molecules (Figure 1-4) could also affect the binding of Siglecs. The negatively charged carboxyl group of Sias is required for recognition by most Siglecs. A requirement of the glycerol-like side chain of Sias at C7-C9 for Siglec binding so far seems to be a general rule (Barnes, Skelton et al. 1999; Angata and Varki 2000; Angata

and Varki 2000; Brinkman-Van der Linden and Varki 2000) with exceptions such as Siglec-6 (Brinkman-Van der Linden and Varki 2000) and Siglec-11 (Angata, Kerr et al. 2002).

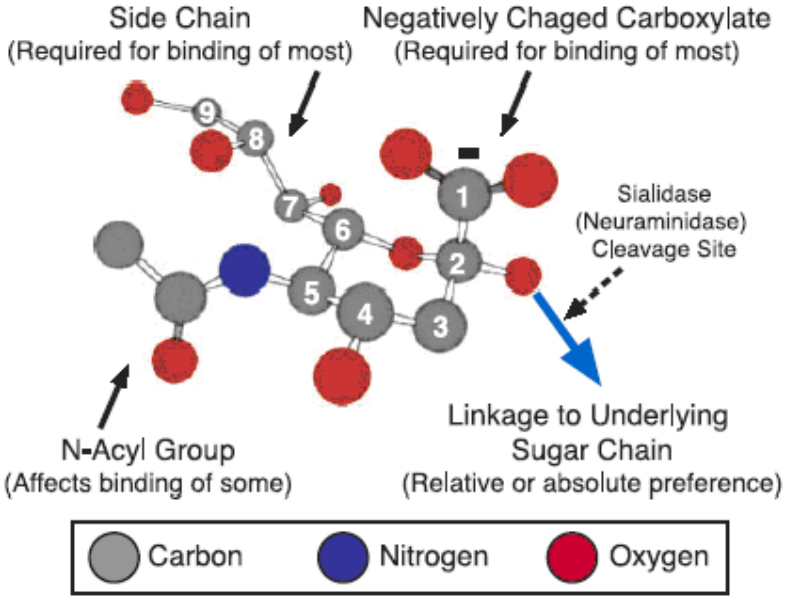


Figure 1-4. Structural features of sialic acids (Sias) affecting recognition by Siglecs. The most common Sia (Neu5Ac) is depicted with the nine carbon atoms numbered. The figure points to various structural features of Neu5Ac (and other Sias) that are known to affect recognition by Siglecs. The site of action of sialidases (neuraminidases) is also shown. (Varki, 2006)

1.1.4.4 The interaction of Siglecs and sialosides

It is of high significance that Siglecs can interact with ligands both in cis and in trans (Figure 1-5). Most Siglecs are masked at the cell surface owing to cis interactions with abundantly expressed sialic acids on the same cell (Freeman, Kelm et al. 1995; Hanasaki, Varki et al. 1995; Razi and Varki 1998). This interaction with cis ligands may dominate over interactions with trans ligands in modulating the biological activities of Siglecs (Crocker 2005). One exception to this rule is sialoadhesin/Siglec-1, which owes to its extended structure (16 V-set Ig domains), is thought to project its sialic-acid-binding site away from the plasma membrane and reduces its cis interactions (Munday, Floyd et al.

1999).

Despite the likely importance of cis-ligand interactions in Siglec function, they do not necessarily prevent the binding of ligands in trans. Following exposure of cells to sialidase, which cleaves the cis-interacting Siglec ligands (Figure 1-4), or in some cases following cellular activation, Siglecs become unmasked, which allows them to make interactions with ligands in trans. Even when Siglecs are masked by cis interactions, trans interactions might occur during an encounter with another cell or a pathogen expressing higher affinity ligands that can out-compete the cis interactions.

The most extensively characterized CD22/Siglec-2 on B cells serves as a good example to illustrate this. In B cells, owing to the interaction of CD22 in cis with sialic acids, CD22 is largely inaccessible to soluble, multivalent sialoside probes, in another word, CD22 is "masked". However, the access to the CD22 receptor can be restored (unmasking) to bind ligands expressed on another cell (Collins, Blixt et al. 2004) when sialic acids are removed by sialidase treatment or in mice lacking the sialyltransferase ST6GAL1, which transfer sialic acids to galactose in α2-6 linkages (Collins, Blixt et al. 2002). Moreover, high-affinity synthetic sialoside probes can out-compete cis ligands for binding to CD22 on native B cells (Collins, Blixt et al. 2006). These results show that cis ligands down-regulate, but do not preclude, binding of ligands in trans, and that equilibrium-based binding of Siglecs to trans ligands can occur dynamically in the presence of cis ligands.

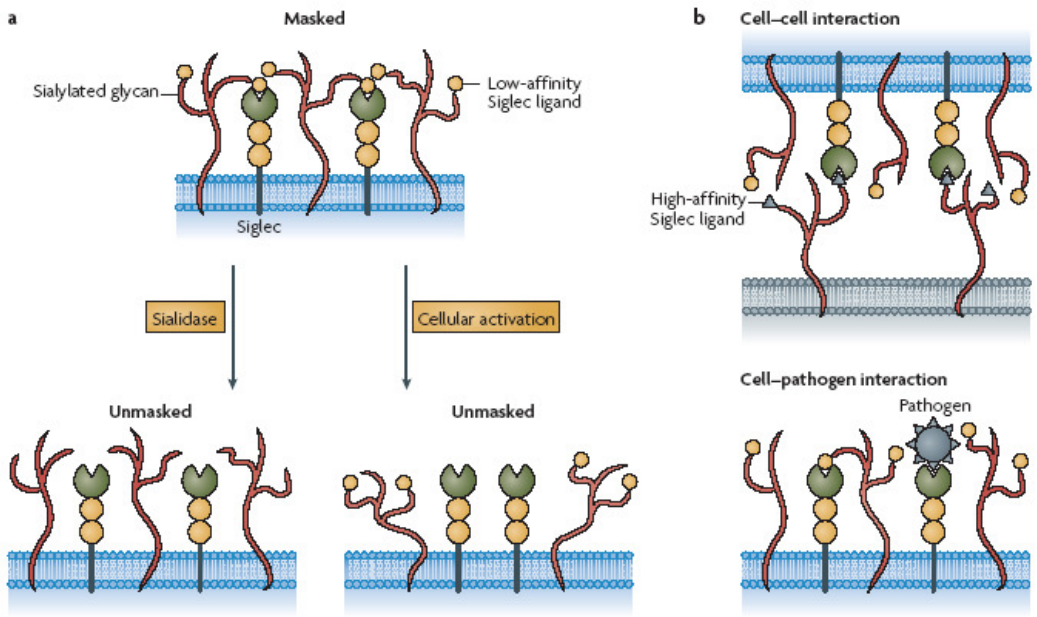


Figure 1-5. Cis and trans interactions of Siglecs. a. Most Siglecs are masked at the cell surface owing to cis interactions with abundantly expressed sialic acids. Following exposure of cells to sialidase, which cleaves the cis-interacting Siglec ligands, or in some cases following cellular activation, Siglecs become unmasked, which allows them to make interactions with ligands in trans. b. Even when Siglecs are masked by cis interactions, trans interactions might occur during an encounter with another cell or a pathogen expressing higher affinity ligands that can out-compete the cis interactions. (Crocker, 2007)

1.1.4.5 Siglec recognition of other specific macromolecules

Several studies have identified other specific ligands (or "counter receptors") for Siglecs. These can be classified into ligands that interact with Siglecs via the sialylated glycans expressed on them and those interact independent of glycans, that is, via protein-protein interactions.

For example, Sn/Siglec-1 was shown to be a counter receptor for the mannose receptor (a macrophage lectin) and the macrophage Gal-binding lectin (Martinez-Pomares, Crocker et al. 1999). The interaction was dependent on sulfated glycans on Sn, which served as a large carrier of glycan ligands for these lectins, rather than as Sia-binding Siglec (Fiete, Beranek et al. 1998).

On the other hand, CD22/Siglec-2 was found to associate efficiently with IgM and CD45 at the surface of B cells independently of sialic acid recognition, despite the fact that these

proteins carry α2-6-linked sialic acids recognized by CD22 (Zhang and Varki 2004). Siglec-6 was also reported to interact with leptin independent of leptin glycosylation (Patel, Brinkman-Van der Linden et al. 1999). However, there has been no definitive report so far on glycan-dependent spedific-binding partner(s) for CD33-related Siglecs.

1.1.5 Siglecs and intracellular signaling

With the exception of a few ones, Siglecs generally have conserved immunoreceptor tyrosine-based inhibitory motif (ITIM) and/or ITIM-like motif in their cytosolic tails. The ITIMs are characterized by a typical 6-amino acid sequence described as (I/L/V) xYxx(L/V), where x denotes any amino acid (Vely and Vivier 1997). Once phosphorylated by a Src-family tyrosine kinase, this motif can interact with the Src homology domain 2-containing phosphatases 1 (SHP-1, also known as protein tyrosine phosphatase (PTP)-1C or PTPN6) and SHP-2 (also known as PTP-1D or PTPN11), as well as with the SH2-domain-containing inositol polyphosphate 5-phosphatase (SHIP) (Figure 1-6). Transmembrane proteins with this motif in their cytoplasmic domains are generally considered to have inhibitory functions, dampening activating signals emitted by other cellular receptors with immunoreceptor tyrosine-based activatory motifs (ITAMs, with typical motif described as YxxLx6-8YxxL).

In contrast, mouse CD33/Siglec-3 and Siglec-H, and human Siglec-14, Siglec-15, and Siglec-16 lack ITIM motifs. But they have a positively charged residue within the transmembrane region that is required to bind to the ITAM-containning adaptors, such as DAP12 and the Fc receptor γ-chain (Tomasello and Vivier 2005). These Siglecs thus might deliver activating signals through ITAM-dependent pathways (Figure 1-6).

1.1.6 Function of Siglecs in the immune system

In general, the most widely accepted explanation for the function of Siglecs is the detection of the “self sialome” and down regulation of the immune system via their ITIM motifs. Numerous studies point to important roles of CD33-related Siglecs in modulating leukocyte

behaviour, including inhibition of cellular proliferation (Vitale, Romagnani et al. 1999; Balaian, Zhong et al. 2003) induction of apoptosis (Nutku, Aizawa et al. 2003; von Gunten, Yousefi et al. 2005), inhibition of cellular activation (Paul, Taylor et al. 2000; Ulyanova, Shah et al. 2001; Avril, Floyd et al. 2004; Ikehara, Ikehara et al. 2004; Avril, Freeman et al. 2005), induction of proinflammatory cytokine secretion (Lajaunias, Dayer et al. 2005) and, in the case of Siglec-H on plasmacytoid dendritic cells (pDCs), suppression of interferon-α (IFNα) production (Blasius, Cella et al. 2006) (Figure 1-6). CD33-related Siglecs can also function as endocytic receptors that could be important in the clearance of sialylated antigens and/or in promoting or inhibiting antigen presentation (Lock, Zhang et al. 2004; Walter, Raden et al. 2005; Nguyen, Ball et al. 2006; Zhang, Raper et al. 2006; Biedermann, Gil et al. 2007). Other functions that are well defined including the contribution for sialoadhesin in the pro-inflammatory functions of macrophages, CD22 as a regulator of B-cell signaling, homeostasis and survival by helping to set a threshold for antigen-induced activation of B cells (Doody, Justement et al. 1995).

In addition, sialoadhesin and several CD33-related Siglecs can interact with sialic acids on Neisseria meningitidis, Campylobacter jejuni, group B Streptococcus and Trypanosoma cruzi (Jones, Virji et al. 2003; Monteiro, Lobato et al. 2005; Avril, Wagner et al. 2006; Carlin, Lewis et al. 2007). Siglec-dependent uptake of these pathogens could potentially benefit the host by promoting pathogen destruction and antigen presentation.

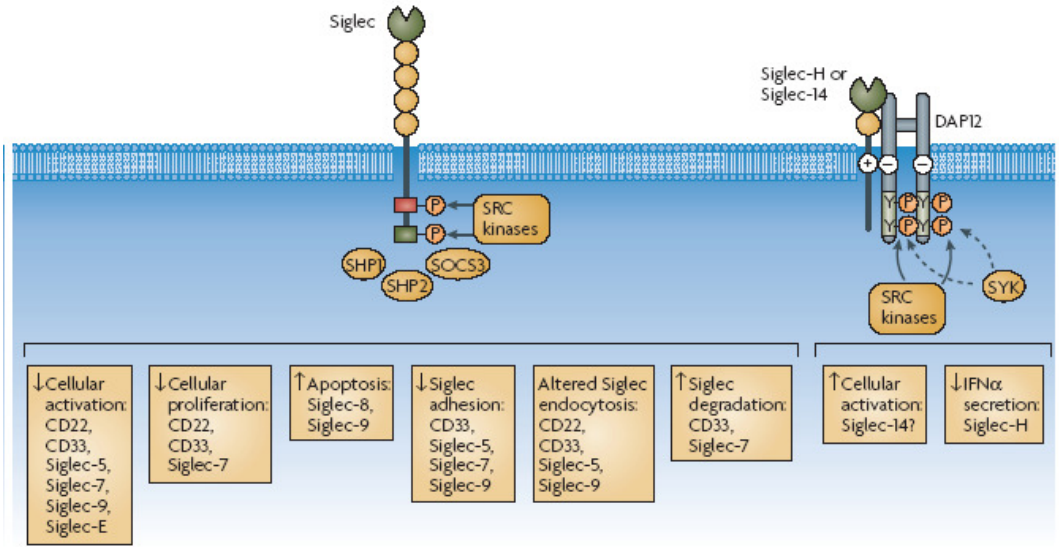


Figure 1-6. Signalling and fuctions mediated by CD22 and the CD33-related Siglecs.

↑, increased; ↓, decreased; IFNα, interferon-α. (Crocker, 2007)

1.1.7 Siglec-11

The recently discovered Siglec-11 belongs to the CD33-related subfamily of Siglecs (Angata, Kerr et al. 2002). The protein deduced from the full-length cDNA of Siglec-11 consists of 5 extracellular Ig-like domains, a single pass transmembrane domain, and a cytosolic tail. Like most of the members of the CD33-related Siglecs, it has immunoreceptor tyrosine-based inhibitory motifs (ITIM) in the cytosolic domain, which have been shown to interact with protein-tyrosine phosphatases SHP-1 and/or SHP-2 (Src homology domain 2-containing phosphatases 1 and/or 2), which are known to be involved in anti-inflammatory signalling of microglia (Horvat, Schwaiger et al. 2001) upon tyrosine phosphorylation (Angata, Kerr et al. 2002). However, Siglec-11 also has several novel features relative to the other CD33-related Siglecs. First, it binds specifically to alpha 2-8-linked sialic acids, but the ligand molecule modified by 2-8-linked sialic acids and recognized by Siglec-11 has not been identified. Second, expression of Siglec-11 was not found on peripheral blood leukocytes, but on tissue macrophages in various tissues, such as liver Kupffer cells and brain microglia.

Siglec-11 is identified as a human-specific gene expressed in microglia (Hayakawa, Angata et al. 2005). Analysis of genome data bases indicated that Siglec-11 has no mouse ortholog. Siglec-11 converted from a pseudogene in humans and chimpanzee, but not in bonobo, gorilla and orangutan. Histopathology demonstrated the expression of Siglec-11 on tissue macrophages in various human tissues, such as liver Kupffer cells, lamina propria macrophages in intestine, microglia in brain, and perifollicular cells in spleen. In inflammatory stomach, the infiltrating cells were also stained intensely. However, the expression of Siglec-11 was not found on peripheral blood leukocytes (Angata, Kerr et al. 2002).

1.2 Microglia

1.2.1 Microglia: parenchymal macrophage of the central nervous system (CNS)

Microglia are one of the glial cells of the CNS. The term "glia" derived from the Greek word for "glue," suggests that microglia share with astroglia and oligodendroglia the property of brain support and, more particularly, the support of neurons. However, such a supportive role in the healthy brain is better appreciated for astroglia, which make important contributions to neurotransmitter metabolism, and for oligodendroglia, which are the source of myelin, than for microglia. In the early 1980s, the macrophagic nature of microglia was formally established (Perry, Hume et al. 1985). Microglia are now known as the major immune cells of the CNS. They reside within the parenchyma of the nervous system sharing many, if not all the properties of macrophages in other tissues. And their role was defined as the first line of immune defense in CNS parenchyma (Kreutzberg 1996).

Although microglia are "brain macrophages," they are distinguished by their parenchymal location, morphology and phenotype from other types of brain macrophages such as meningeal and perivascular macrophages (Polfliet, Zwijnenburg et al. 2001; Nguyen, Julien et al. 2002; Polfliet, van de Veerdonk et al. 2002) and perivascular cells or pericytes (Thomas 1999; Williams, Alvarez et al. 2001), which are enclosed by a perivascular basement membrane within blood vessels and are not part of the CNS parenchyma. In particular, only microglia localize within the CNS parenchyma itself, in close contact with neurons, astrocytes and oligodendrocytes. Interestingly, a subpopulation of microglia, referred to as juxtavascular microglia, directly contacts the basal lamina of CNS blood vessels, at the blood–brain barrier (Lassmann, Zimprich et al. 1991; Gehrmann, Matsumoto et al. 1995). It is noteworthy that, despite their localization along blood vessels, juxtavascular microglia are phenotypically and morphologically distinct from perivascular macrophages (Kida, Steart et al. 1993). Under normal conditions, the adult mouse brain contains an average of 3.5×10^6 microglial cells (Lawson, Perry et al. 1990). On a weight to-weight basis, microglia are thus as numerous as other tissue macrophages such as

Kupffer cells in the liver.

1.2.2 Origin and turnover of microglia

In rodents and humans, postnatal microglia are thought to arise from two different pools of myeloid cells that successively colonize the developing CNS. The first wave of microglial progenitors invades the embryonic and fetal CNS and derives essentially from extramedullary sources of hematopoiesis, including the yolk sac (Rezaie and Male 1999; Kaur, Hao et al. 2001). The second wave of microglial progenitors is formed by bone marrow (BM)–derived monocytic cells that colonize the CNS during the early postnatal period (P0–P15) in rodents, or before birth in humans (Cuadros and Navascues 1998). But the precise identity of these monocytic cells is yet not formally established. Passed the late phases of CNS development, the traffic of leukocytes from blood to CNS parenchyma is exquisitely controlled by the blood–brain barrier (BBB) (Bechmann, Galea et al. 2007). In the adult brain, resident microglia have a slow turnover at rest and are capable of proliferation and self-renew. Thus, the population of microglia in the adult under normal conditions is replenished intrinsically and does not require significant turnover from circulating blood progenitors (Lassmann and Hickey 1993; Kennedy and Abkowitz 1997).
However, numerous reports showed that bone marrow stem cells (BMSCs) have the ability to populate the CNS and differentiate into functional parenchymal microglia as well as perivascular macrophage (Priller, Flugel et al. 2001; Priller, Persons et al. 2001; Vallieres and Sawchenko 2003; Simard and Rivest 2004; Massengale, Wagers et al. 2005). Even though BMSCs can enter the brain parenchyma throughout the CNS in normal mice, it seems that they are preferentially attracted to regions afflicted by neurodegeneration or neurological insults. In the case of cerebral ischemia, round donor-derived cells (most likely blood monocytes) enter the brain at the site of injury, and then migrate from the infiltration site and become ramified microglial cells. This is also true in models where the BBB is not compromised, such as in the case of facial nerve axotomy and hypoglossal nerve axotomy. It is reported that prion neuroinvasion is accompanied by a major recruitment of BM-derived microglia. Indeed, more than 50% of all brain microglia were replaced by

BM-derived cells before clinical disease onset and that in terminally sick mice, microglia density increased threefold to fourfold. These findings suggest that blood monocytes infiltrate the brain and later differentiate into ramified microglia, and that they are able to enter the CNS even if the BBB is intact and they can massively colonize the CNS in particular diseases. More importantly, these cells are recruited as a consequence of the disease and are not involved in the progression of the neuropathology (e.g. prion neuroinvasion). However, the exact mechanisms regulating microglia homeostasis remains a subject of debate.

1.2.3 Role of microglia in the CNS

The most characteristic feature of microglia is their rapid activation in response to pathological change in the CNS. They respond not only to changes in the brain parenchymal integrity but also to very small alterations in their microenvironment, such as imbalances in ion homeostasis that precede pathological changes (Gehrmann and Kreutzberg 1993).

In the normal mature brain, microglia typically exist in a resting state characterized by ramified morphology, and monitor the brain environment by extending their processes over a multitude of nonoverlapping territories that cover the entire neural parenchyma. They are called quiescent microglia in this state. However, under a number of pathological conditions, quiescent ramified microglia will activate and engage a series of morphological alterations that lead to a hypertrophy of microglia cell body and a retraction of their ramifications. By the end of such a process, fully activated microglia, also called reactive microglia, harbor a similar morphology than any activated macrophage. Activated microglia were found to exert functions commonly assigned to all tissue-resident macrophages under inflammatory conditions. These include notably phagocytosis (Bauer, Sminia et al. 1994), antigen presentation (Perry 1998) and secretion of proinflammatory cytokines such as interleukin 6 (IL-6), IL-1 or tumor necrosis factor α (TNF-α) (Banati, Gehrmann et al. 1993).

The outcomes of the microglial activateion towards harmful or beneficial effect depend on the activating conditions. On the one hand, they have a critical role in host defense by removing invading microorganisms and neoplastic cells, or by secreting neurotrophic

factors. On the other hand, microglia may aggravate the effects of inflammation and cause neuronal degeneration. Over activated microglia could damage or induce apoptotic death of neurons, either directly through the release of toxic mediators such as cytokines and free radicals or indirectly by attracting activated T cells, monocytes, and neutrophils into the CNS. It is generally accepted that activated microglia function as a "double-edged sword," with neuroprotective features predominating in the healthy nervous system and neurodestructive properties observed in various disease states such as in Alzheimer's disease (AD), Parkinson's disease, and Huntington's disease etc (Stoll and Jander 1999; Hanisch and Kettenmann 2007).

1.2.4 Molecules and signaling pathways involved in microglial activation

LPS (abd-el-Basset and Fedoroff 1995; Kim and Joh 2006), an endotoxin from the gram-negative bacterial cell wall, is a potent immunostimulantor of microglia. Its recognition involves the binding of LPS to the serum protein LBP (LPS binding protein) and transfer of the complex by CD14 to the cognate receptor toll-like receptor 4 (TLR4) and the accessory protein MD-2. A variety of intracellular signaling molecules, such as protein tyrosine kinases, nitrogen-activated protein kinases, protein kinase C, small G proteins, and ceramide-activated protein kinase are involved in LPS-mediated activation. Through different signal transduction pathways, LPS activates transcription factors including NF-κB, NF-IL6, C/EBP and Fos/Jun families, and induces cytokine genes such as induced nitric oxide synthase (iNOS), TNF-α, IL-1β, IL-6, transforming growth factor β (TGF-β) (Sweet and Hume 1996).

Interferon γ (IFN-γ), released from activated Th1 and NK cells, activates microglia to increase expression of MHC class I and class II molecules. With LPS, it synergistically induces IL-12 production from microglia. IFN-γ-mediated activation involves the JAK-STAT pathway. Briefly, IFN-γ stimulates the activation of receptor associated Jak1 and Jak2. This leads to the phosphorylation of a single receptor tyrosine residue, which is then recognized by the SH2 domain of Stat. It causes Stat phosphorylation followed by homodimerization, translocation into nucleus and induction of GAS (gamma-activation site)

driven target genes (Schindler 1999).

Chemokines are small proteins (8 to 10 kDa) that induce chemotaxis, tissue extravasation and functional modulation of a wide variety of leukocytes during inflammation (Taub 1996). More than 40 distinct members are divided into 4 families typified by conservation of cysteine residues in the N-terminal sequence (Lusti-Narasimhan, Chollet et al. 1996). Chemokines mediate their effects via G protein-coupled receptors of the seven transmembrane domains. A number of chemokines are expressed in the CNS. They are related to a number of diseases of the CNS including stroke, multiple sclerosis (MS) and AD. Fractalkine/ neurotactin is a unique member of CX3C chemokine family which was discovered in 1997 (Bazan, Bacon et al. 1997). In the CNS, several populations of neurons express fractalkine mRNA constitutively that is not affected by stimuli such as cytokines, LPS and toxic stimuli (Amyloid β, glucose deprivation or glutamate). Membrane-bound fractalkine protein levels were decreased after excitotoxic glutamate stimuli (Chapman, Moores et al. 2000). Its receptor, CX3CR-1 is expressed at high levels in microglia (Nishiyori, Minami et al. 1998). Through its receptor, fractalkine induces intracellular Ca^{2+} mobilization, ERK activation and PI3-K-mediated PKB activation in microglia.

CD40 is a 45-50 kDa transmembrane protein, which is a member of the TNFR (tumor necrosis factor receptor) superfamily (Vogel and Noelle 1998). It has been shown that CD40 is constitutively expressed at low levels on microglia, and binding of microglial CD40 by CD40 ligand (CD40L) leads to marked TNF- secretion, which is neurotoxic at such levels (Aloisi, Penna et al. 1999). Activation of ERK1/2 is involved in CD40-CD40L mediated microglial activation (Tan, Town et al. 2000). Interestingly, stimulation with Amyloid β peptides (Aβ) and CD40L results in increased CD40 expression on microglia followed by TNF-α secretion. It has also been demonstrated that CD45 suppresses CD40L-induced microglial activation via negative regulation of the Src/ERK1/2 cascade.

Aβ is the principal component of the extracellular deposits in AD (Selkoe 1989). Aβ promotes neurite outgrowth, generates reactive oxygen intermediates, induces cytotoxic cellular oxidative stress, and microglial activation (Koo, Park et al. 1993; Behl 1997; Sasaki, Yamaguchi et al. 1997). Although the mechanism by which Aβ causes enhanced expression of proinflammatory cytokines from microglia is not fully understood, there is evidence that

Aβ may interact with cell-surface receptors, including receptors for advanced glycosylated endproducts and scavenger receptors (El Khoury, Hickman et al. 1996; Yan, Chen et al. 1996). Additionally, calcium, protein kinase C, and protein tyrosine kinase-dependent second messenger pathways have been postulated in Aβ receptor-mediated signal transduction (Lorton 1997; Combs, Johnson et al. 1999). Aβ activates microglia through these signal transduction pathways to induce the secretion of neurotoxic substances including TNF-α and IL-1, enhancing likely neuroinflammation in AD brain (Mrak and Griffin 2001; Smits, de Vos et al. 2001).

Gangliosides, the sialic acid-containing glycosphingolipids, have also been reported as microglial activators (Pyo, Joe et al. 1999). Gangliosides exist in mammalian cell membranes and are particularly rich in the neuronal cell membrane. Gangliosides induce production of nitric oxide (NO), TNF- and cyclooxygenase-2 (COX-2) in microglia by activation of MAPKs (mitogen-activated protein kinases). Studies show that signals are released from neurons when they start to die. Upon potassium deprivation, cerebellar granule cells release signal molecules that can activate microglia (Tanaka, Suzuki et al. 1998). Supernatant from serum-deprived immortalized motor neurons can also activate microglia and induce release of NO that causes neuronal death. These signals from dying neurons may be potent candidates for microglial activation.

Thrombin-mediated microglial activation has been reported (Moller, Hanisch et al. 2000). Thrombin is generated from the precursor prothrombin that is endogenously expressed in human, mouse, and rat brain, including dopaminergic neurons in the CNS (Dihanich, Kaser et al. 1991; Soifer, Peters et al. 1994; Weinstein, Gold et al. 1995). Thrombin-induced microglial activation involves protease-activated receptor-1 (PAR-1) (Suo, Wu et al. 2002). Studies demonstrated that direct injection of thrombin into various brain regions including hippocampus and substantia nigra results in induction of iNOS, COX-2 and NADPH oxidase-mediated superoxide generation from microglial and subsequent neuronal degeneration (Choi, Lee et al. 2003; Choi, Lee et al. 2005).

1.3 Lentiviral vector system

1.3.1 General concept of viral vectors

Viruses are dependent on their host cell to carry their genome. They are intracellular parasites that have developed efficient strategies to invade host cells and, in some cases, transport their genetic information into the nucleus either to become part of the host's genome or to constitute an autonomous genetic unit. Viral vectors are the widely used vehicles developed from some natural virus to deliver genes to target cells. Viral vector comprises the viral sequences that are required for the assembly of viral particles, the packaging elements that can package the viral genome into the particles, the cassettes that are required to deliver the gene of interest (also termed transgene) to the target cells, and the transgene. Dispensable genes from the viral genome are deleted to reduce patho- and immunogenicity.

Viral vectors can be divided into two general categories (Pfeifer and Verma 2001): (a) integrating vectors, capable of providing life-long expression of the transgene, and (b) non-integrating vectors. Examples for integrating vectors are retroviral and adeno-associated virus (AAV)–derived vectors. The major non-integrating vector currently employed is based on adenoviruses, and the viral DNA is maintained as an episome in the infected cell. Each of these vectors has specific advantages and major limitations. It is accepted that an ideal vector should fulfill the following requirements (Somia and Verma 2000):

1. Efficient and easy production. High-titer preparations of vector particles should be reproducibly available. The efficient transduction of cells is only possible if a sufficient number of infectious particles reach the target cells. For the widespread use of viral vectors, facile production procedures have to be developed.

2. Safety aspects. The vector should neither be toxic to the target cells nor induce unwanted effects, including immunological reactions against the viral vector or its cargo. The latter carries not only the threat of eliminating the vector and/or the infected cells but also may lead to life-threatening complications, such as septic shock.

3. Sustained and regulated transgene expression. The gene delivered by the viral vector has to be expressed in a proper way. Permanent or even life-long expression of the therapeutic gene is desired only in a minority of diseases (e.g., treatment of hemophilia). Controlled expression of the transgene in a reversible manner would be highly desirable in many cases.
4. Targeting of the viral vectors. Preferential or exclusive transduction of specific cell types is very desirable.
5. Infection of dividing and nondividing cells. Because the majority of the cells in an adult human being are in a postmitotic, nondividing state, viral vectors should be able to efficiently transduce these cells.
6. Site-specific integration. Integration into the host genome at specific site(s) is especially helpful in gene targeting.

1.3.2 Constitution of lentiviral vectors

Lentiviruses belong to complex retroviruses, a group of RNA virus. The term “lenti” derives from “lente” in Latin, which means slow. Two outstanding features of lentiviruses make them a very attractive tool for gene delivery. The first is their ability to infect nondividing, terminally differentiated mammalian cells. HIV (human immunodeficiency virus)-derived lentiviral vectors transduce a broad spectrum of nondividing cells in vivo and in vitro, such as neurons (Naldini, Blomer et al. 1996), retinal cells (Miyoshi, Blomer et al. 1998; Takahashi, Miyoshi et al. 1999), muscle cells (Kafri, Blomer et al. 1997), and hepatocytes (Pfeifer, Kessler et al. 2001). And the second is the ability to efficiently deliver large (»8 kb) and complex transgenes to the target cells and tissues (Trono 2000).
Lentiviral vectors have been derived from HIV-1 (Naldini, Blomer et al. 1996; Poeschla, Corbeau et al. 1996; Reiser, Harmison et al. 1996), HIV-2 (Poeschla, Gilbert et al. 1998), feline immunodeficiency virus (FIV) (Poeschla, Wong-Staal et al. 1998), equine infectious anemia virus (Olsen 1998), simian immunodeficiency virus (SIV) (Mangeot, Negre et al. 2000), and maedi/visna virus (Berkowitz, Ilves et al. 2001). But most of the lentiviral vectors presently in use for gene therapy approaches are HIV-derived vectors.
To increase safety in practice, the lentiviral vector system is divided into vector constructs

and helpful packaging vectors (Figure 1-7). Cis- and trans-acting factors of lentiviruses are separated into different plasmids while preserving their functions. The vector constructs contain the viral cis elements, packaging sequences (ψ), the Rev response element (RRE), the central polypurine tract (cPPT), and the transgene and its expression regulatory elements, while the lentiviral packaging systems provide in trans the viral proteins that are required for the assembly of viral particles in the packaging cells.

The long terminal repeats (LTRs) are viral sequences containing many cis-acting control elements for reverse transcription of the vector RNA and integration of the proviral DNA. The LTRs are divided into the U3, R, and U5 regions. In lentivirus, the U3 region in the 5' LTR is replaced with the immediate early region of the human cytomegalovirus (CMV) enhancer-promoter. The CMV/LTR hybrid has a high transcriptional activity, especially when introduced in the appropriate cell lines (Finer, Dull et al. 1994), e.g., human embryonic kidney (HEK), 293 cells. This cell line expresses the adenoviral E1 gene products (Graham, Smiley et al. 1977) that superactivate the CMV promoter (Gorman, Gies et al. 1989). The 3' LTR contains the cis-acting control elements involved in posttranscriptional processing of the 3' end of the viral RNA (e.g., polyadenylation). The promoter/enhancer sequences of the U3 region of the 3'LTR is deleted or mutated so that the viral vectors are self-inactivating (SIN). This could avoid the problem of insertional activation of cellular oncogenes through the promoter and enhancer elements of the proviral LTR.

The packaging sequences (ψ) are required for encapsidation of the vector RNA. The Rev response elements (RRE) could be recognized by the Rev protein of HIV which promotes the efficient transport of unspliced RNAs containing RRE from the nucleus to the cytoplasm. The central polypurine tract (cPPT) is to enhance nuclear translocation of the vector in the target cell.

The transgene in lentiviral vectors is normally regulated by internal promoters such as CMV promoter or other tissue specific promoter to restrict the expression to a specific cell type or tissue. In addition, this approach allows the incorporation of regulatable transcriptional elements that may be switched on and off via exogenous stimuli, for example the tetracycline-regulated system, in which the transgene expression is induced in a

tet-dependent manner. Another example is the inclusion of Flap elements, which could work together with Cre to regulate the expression of transgene.

An important improvement of the lentiviral vectors compared to other retroviral vectors is the inclusion of cis-acting transcriptional regulatory elements, such as the WPRE (wood chuck hepatitis virus post-transcriptional regulatory element), which enhances transgene expression in the target cells. The WPRE has to be present within the transgene transcript in sense orientation and is placed 3' of the transgene cDNA upstream of the 3' LTR.

To package the replication-defective vector into virions, the necessary viral proteins are provided in trans in the packaging cell. Studies on HIV-1 demonstrated that structural components of lentiviruses can be provided in trans by packaging plasmids, and viral particles can be assembled by expressing viral proteins in packaging cells. The third generation of lentiviral packaging constructs includes three plasmids. One plasmid carries gag, pol (the two necessary viral gene), and the HIV RRE. A separate expression plasmid encodes Rev, which facilitates the expression of gag and pol. The third plasmid incorporates the G protein of the vescular stomatitis virus (VSV-G). The major advantages of incorporation of the VSV-G protein are (a) the extremely broad host range of VSV, which enters the host cell by membrane fusion via the interaction with phospholipid components of the cell membrane (Mastromarino, Conti et al. 1987) and (b) the ability to concentrate VSV-G pseudotyped particles more than 1000-fold (titers $>10^9$ IU/ml) by ultracentrifugation (Burns, Friedmann et al. 1993), which has important practical implications. Splitting the packaging genome into multiple units not only increases the safety of lentiviral vectors but also facilitates pseudotyping of lentiviral vectors with the envelope of different viruses.

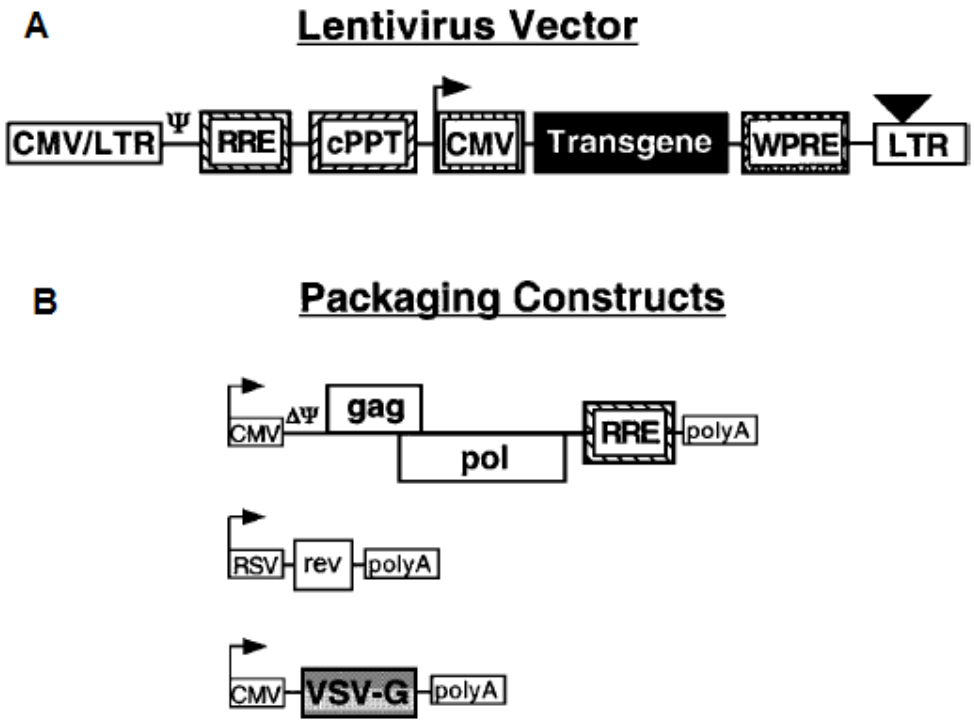


Figure 1-7. Sketch of lentivial vectors system. A. key components of lentiviral vectors. B. sketch of packaging vectors. (Pfeifer and Verma 2001)

1.4 Transgenic mice

1.4.1 Transgenic mouse as an invaluable model

A transgenic animal is one that carries a foreign gene that has been deliberately inserted into its genome. The ability to introduce and express exogenous genes of interest in animals has become an indispensable tool to modern biologists (Jaenisch 1988). Using transgenic techniques, a characterized genetic sequence may be evaluated within the specific genomic background of the whole animal. Currently the most common uses of transgenic animals are (1) for studies of tissue-specific and developmental-stage-specific gene regulation and (2) for experiments of the phenotypic effects of transgene expression. Among the experimental animals, mouse is chosen as a widely used model for good reasons (Gondo 2008). Not only because it is closely related to humans but also because it has more than 100 years of history

in genetic analysis. Over this period many mutants were identified, a number of inbred lines were established and gene mapping had been conducted more extensively than in any other mammalian species. Mouse exhibits a short life span with the large litter size that is suitable for genetic studies. In addition, mouse is currently the only species for which embryos can be manipulated using available ES-cell (embryonic stem cell) technologies. Furthermore, technologies for freezing embryos and gametes are well established in mouse, allowing in vitro fertilization to be combined with embryo transfer methods. Thus, valuable mouse lines can be easily and stably maintained in liquid nitrogen for many years while requiring minimal space and manpower.

Plenty of technologies have been developed to control the expression of interested genes and facilitated the generation of transgenic mouse, among them are gene targeting (Smithies, Gregg et al. 1985; Wong and Capecchi 1986; Capecchi 2005) including knockout and knockin, specific expression of trangene using tissue specific promoters, introduction of dominant negative mutations to eliminate the activities of the wild-type gene products, insertion of a transgene as a mutagen, and disruption of the gene functions by RNA interference. Furthermore, the employment of inducible regulation approaches (Lewandoski 2001), such as the Cre/loxP (Akagi, Sandig et al. 1997), the Flp/Frt (Theodosiou and Xu 1998) and the tetracycline system (Berens and Hillen 2003), have greatly expanded the spectrum of transgenic mice. In cases where mutations could provoke lethality during development or invalidation of wildly expressed genes might lead to a complex phenotype affecting multiple tissues, it was limited to create mouse carrying such kinds of mutations. However, when applying inducible systems, the expression of such mutations could be rendered conditional, thus make it possible to generate mouse expressing the transgenes or mutations only in a specific time period or in one of the interested tissue.

Two methods of producing transgenic mouse are widely used, one is injecting the desired gene into the pronuclear of a fertilized mouse egg (Rulicke and Hubscher 2000), and the other is using transformed ES cells with the desired DNA (Robertson 1991).

1.4.2 Generation of transgenic mouse through pronuclear injection

The pronuclear microinjection method (Rulicke and Hubscher 2000) is the most extensively and successfully used method of gene transfer in mouse. It means microinjection of a purified double-stranded DNA sequence directly into the pronuclei of fertilized zygotes to produce a transgenic animal. If this transgene is integrated into one of the embryonic chromosomes, the animal will be born with a copy of this new information in every cell. The animal that develops after receiving the transgene DNA is referred to as the founder (F0) of a new transgenic lineage. If the germ cells of the founder transmit the transgene stably, then all descendants of this animal are members of a unique transgenic lineage. A homozygous genotype may be produced by the mating of a pair of hemizygous F1 siblings (Fig 1-8).

Despite the relatively simpleness, this method has some shortcomings. Firstly, integration of foreign DNA into the embryonic genome generally is a random event with respect to the chromosomal locus. Therefore the probability of identical integration events in two embryos receiving the same transgene is overwhelmingly unlikely. Secondary, it is impossible to regulate exactly how many copies of the transgene will be introduced into the embryo and how many will join together to integrate. In addition, the transgene can insert into functional endogenous genes and interrupt the normal expression of them, which may be inconsequential or lethal. Alternatively, observable insertional mutagenesis might be apparent when the insertion interferes with the expression of an endogenous developmentally active gene. Thus the identification of the locus of transgene insertion is of great value when analyzing these transgenic animals.

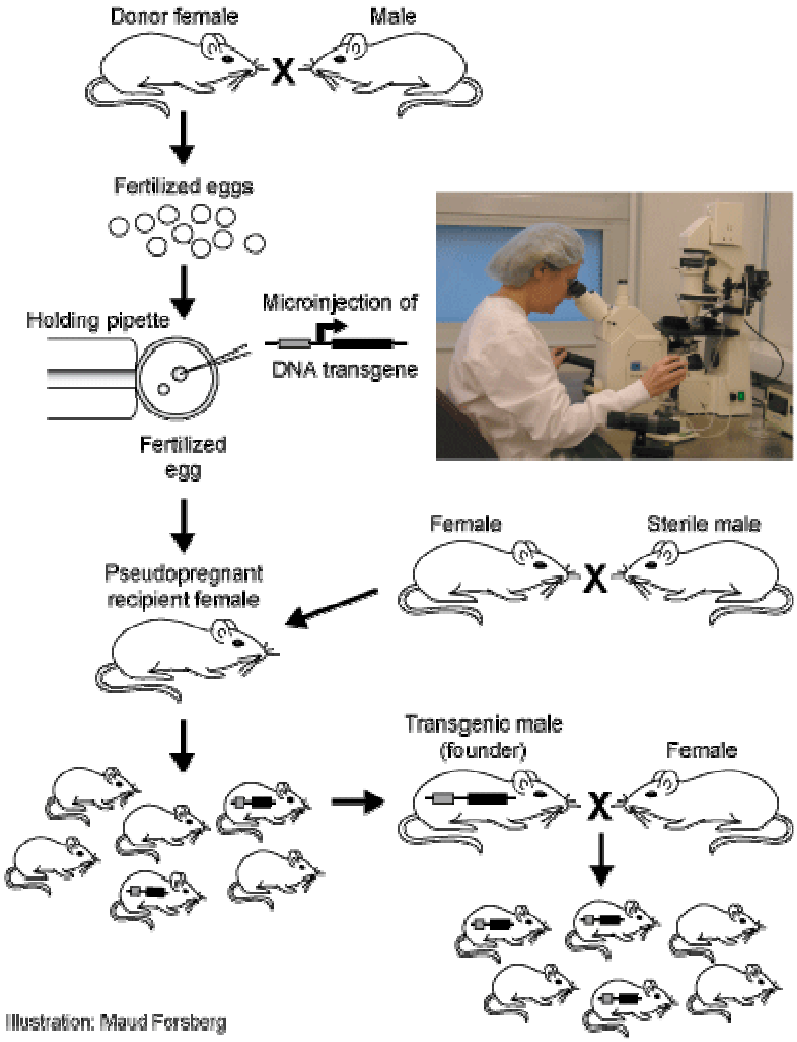


Fig 1-8. Generation of transgenic mice through pronuclear injection. The construct containing a promoter, the target transgene and a poly A sequence is microinjected to the pronuclei of zygotes from donor mice. The injected embryos are transplanted into pseudopregnant foster mothers. Fonders are verified and further breed to establish transgenic lines. (From http://www.imbim.uu.se)

1.4.3 ES cell-mediated transgenic mice

ES cells are pluripotent cells derived from the inner cell mass of blastocyst-stage embryos. They can be maintained in culture as undifferentiated under the proper growth conditions. A broad spectrum of strategies has been designed to create genomic alterations in these cells. Homologous recombination-based gene targeting, heterologous site-specific recombinases (Cre recombinase, Flp recombinase), positive and negative selectable markers,

reporters, and the availability of the mouse genome sequence have created an arsenal of tools that allow tailoring the mouse genes and genomes at will. When the genetically altered ES cells are injected into a host blastocyst, or aggregated within a morula-stage embryo, they have the capacity to contribute to all tissues of the resultant chimeric mouse or fully ES cell–derived F0 generation mouse (Poueymirou, Auerbach et al. 2007) (Figure 1-9). Most important, ES cells can contribute to germ cells and transmit the genetic mutations in vivo, allowing development of established mouse lines in which the altered gene(s) are carried.

ES cell-mediated transgenesis has several advantages over the standard pronuclear DNA injection. ES cells make the site specific gene targeting possible. They provide a higher frequency of low-copy numbers or even single copy of transgene integration. In addition, modified ES cell clones can be tested in vitro for cell type specific expression by ES cell differentiation assays.

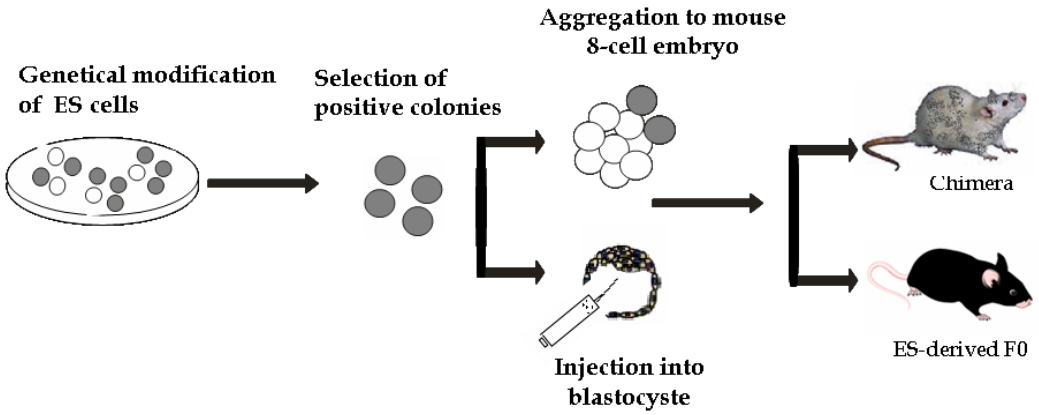


Figure 1-9. Production of trangenic mice by ES cell-mediated methods. Genetically modified ES cells were aggregated to the 8-cell embryos or microinjected into the blastocysts. Chimeras could derive from both ways. But it is also possible to generate F0 generation mice fully derived from ES cells by laser assisted injection of ES cells to 8-cell embryos.

1.4.4 The Cre/loxP system

The Cre protein is a recombinase identified in the P1 bacteriophage, which reacts when it recognizes a sequence of 34 base pairs (called loxP) in a segment of DNA (Kilby, Snaith et al. 1993) (Figure 1-10). When two loxP sites are oriented in the same direction, the Cre recombinase induces the deletion of the DNA segment placed between them. Conversely, if

the loxP sites are oriented in opposite direction, recombination induces its inversion. Cre recombinase activity does not require a DNA co-factor or particular topology. Moreover, it is active in the eukaryote cells (Sauer and Henderson 1988) in vitro and in vivo.

To take the advantage of the Cre/loxP system to establish conditioned transgenic mice, the first step is to create mice carrying alleles in which two loxP sites surround the gene or sequence to be studied without disrupting its activity. These mice are then crossed with a transgenic mouse expressing the Cre recombinase in a particular cell type. In the resulted offsprings, the Cre recombinase promotes the deletion of the sequences located between the loxP sites and induces a null mutation in the cell type in which the transgene is expressed. So as long as a line of transgenic mice expressing protein Cre in the tissue concerned is available, tissue specific transgenic mice can be easily derived (Tsien, Chen et al. 1996; Shibata, Kanamaru et al. 1997; Kulkarni, Bruning et al. 1999)

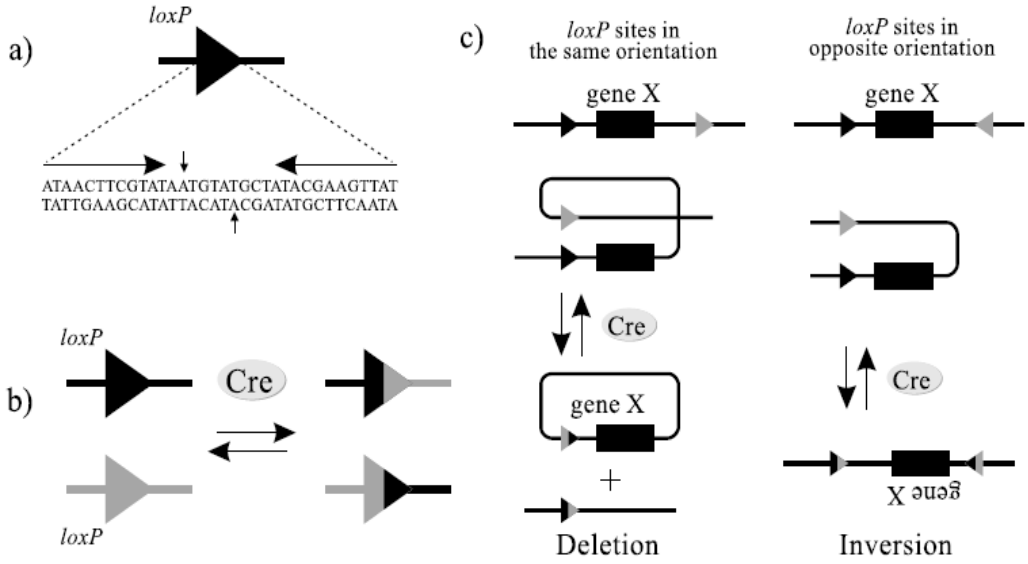


Figure 1-10. The Cre/loxP system and its application. The loxP site, symbolized by a triangle is a sequence of 34 base pairs composed of palindromic sequences of 13 bp separated by a sequence of 8 bp (a). Cre recombinase specifically recognizes this sequence, provokes the cleavage in DNA (vertical arrows, a) and induces the recombination of DNA between the two loxP sites as illustrated in (b). Recombination could result in gene deletion or inversion(c). If the two loxP sites have the same orientation, the DNA region situated between these sites is deleted during recombination. If the orientation of the two loxP sites is opposed, recombination leads to the inversion of the region comprised between the two sites. (Chales Babinet 2001)

1.5 Aim of the study

Siglec-11 is a recently identified CD33-related Siglec. It is identified as a human-specific gene expressed in microglia. When considering the features of the CD33-related Siglec family and the specific expression pattern of Siglec-11 on tissue macrophages, particularly in brain microglia, one can imagine that this evolutionally new Siglec might be developed in humans as an important microglial-specific molecule to create an immunosuppressive milieu in the CNS. Thus, we were asking the following questions: Is Siglec-11 involved in anti-inflammatory signaling in microglia? Does Siglec-11 have anything to do with neuroinflammatory diseases such as multiple sclerosis and Alzheimer's disease?

To reveal the answer to these questions, we set out to study the function of Siglec-11 in microglia. Due to the limitation in acquiring human microglial cell, our functional assay was based on mouse cells. First, we aimed to establish a microglial cell model which expresses Siglec-11 for functional assays in vitro. Second, functional study of Siglec-11 was focused on microglia. Third, the ligand that Siglec-11 might bind was investigated. Fourth, we aimed to generate a transgenic mouse model which expresses Siglec-11 specifically in microglia and macrophages to study the function of Siglec-11 in vivo.

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Buffers and Solutions

10X (0.125M) Phosphate buffered saline (PBS), pH 7.3

Components	Concentration	Company
$NaH_2PO_4*H_2O$	0.007M	Roth, Germany
$NaH_2PO_4*7H_2O$	0.034M	Roth, Germany
NaCl	0.6M	Roth, Germany
ddH_2O	up to 1 liter	Roth, Germany

4% Paraformaldehyde (PFA), pH 7.3

Components	Amount	Company
PFA	20g	Sigma, Germany
NaOH	30ml	Roth, Germany
PBS (10X)	50ml	
ddH_2O	up to 1 liter	Roth, Germany

10X TBE buffer

Components	Concentration	Company
Tris-Base	1.78M	Roth, Germany
Boric Acid	1.78M	Sigma, Germany
EDTA	0.04M	Roth, Germany
ddH_2O	up to 2 liter	Roth, Germany

50X TAE buffer (pH 8.5)

Components	Amount	Company
Tris-Base	242 g	Roth, Germany
Acetic acid	57.1 ml	Sigma, Germany
0.5 M EDTA	100 ml	Roth, Germany
ddH_2O	up to 1 liter	Roth, Germany

6X Loading buffer

Components	Concentration	Company
EDTA	0.5M	Roth, Germany

Sucrose	60%	Roth, Germany
Bromphenol Blue	0.04%	Sigma, Germany
Xylene Cyanole	0.04%	Sigma, Germany
Ficol-400	2%	Bio-Rad, Germany

1% Agarose gel

Components	Amount	Company
Agarose	0.5g	Biozym, Germany
Ethidium Bromide	1.25µl	Roth, Germany
TBE (1X)	50ml	

Reverse transcription (RT) mix

Components	Amount	Company
Total RNA	5µg	
Hexanucleotide Mix (10X)	1µl	Roche, Germany
dNTP mix (10mM)	1µl	Sigma, Germany
DTT mix (10mM)	2µl	Invitrogen, Germany
5X RT 1st Strand Buffer	4µl	Invitrogen, Germany
RT enzyme (200U/ml)	1µl	Invitrogen, Germany
ddH_2O	up to 20µl	Roth, Germany

Real time RT-PCR mix

Components	Amount	Company
cDNA (200ng/µl)	1µl	
SYBR Green Master Mix (2x)	12.5µl	Applied Biosystems
Primer mix (20pmol/µl)	1µl	MWG, Germany
ddH_2O	10.5µl	Roth, Germany

PCR reaction mix (50 µl)

Components	Amount	Company
Buffer (10X)	5µl	Roche, Germany
dNTP mix (10mM)	1µl	Amersham Bioscience, USA
Primer pair mix (20 pmol/ul)	2µl	MWG, Germany
Taq polymerase(100U/20ul)	1µl	Roche, Germany
ddH_2O	Up to 50µl	Roth, Germany

Digestion reaction mix (20 µl sample)

Components	Amount	Company
Buffer (10X)	2µl	Roche, Germany
Enzyme	10 U	Roche, Germany
Plasmid/insert	Up to 1 µg	
ddH_2O	Up to 20µl	Roth, Germany

Ligation reaction mix (20 µl sample)

Components	Amount	Company
Buffer (10X)	2µl	Roche, Germany
T4 Ligase (1U/ul)	4 µl	Roche, Germany
Or T4 Ligase (2000 1U/µl)	1 µl	NEB, Germany
Plasmid and insert fragment	In correct ratio	
ddH_2O	Up to 20µl	Roth, Germany

Protein lysis buffer

Components	Concentration	Company
RIPA buffer	Cat.no.R2078	Sigma, Germany
PMSF	1 mM (174 µg/ml)	Sigma, Germany
Aprotinin	5 µg/ml	Sigma, Germany
Leupeptin	5 µg/ml	Sigma, Germany
Phosphatase inhibitors		Sigma, Germany
Na_3VO_4		NEB, Germany

Buffers for SDS-PAGE and Western Blot

Components	Cat. No	Company
NuPAGE LDS Sample Buffer (4x)	NP0007	Invitrogen, Germany
NuPAGE® MES SDS Running Buffer (20X)	NP0002	Invitrogen, Germany
NuPAGE® Tris-Acetate SDS Running Buffer (20X)	LA0041	Invitrogen, Germany
NuPAGE® Transfer Buffer (20X)	NP0006	Invitrogen, Germany
BenchMark® Protein Ladder	10747-012	Invitrogen, Germany
SeeBlue® Pre-Stained Standard	LC5625	Invitrogen, Germany

PBS-Tween-20 (PBST)

Components	amount	Company
Tween-20	500 µl	
1x PBS	1000 ml	

Membrane blocking solution

Components	concentration	Company
BSA	3%	Sigma, Germany
Or Milk powder	5 %	Sigma, Germany
PBST	100 ml	

2.1.2 Cell culture media and reagents

DMEM medium (for MEFs, 293FT cells)

Components	Concentration	Company
DMEM, high glucose		Gibco, Germany
Fetal bovine serum	10%	Gibco, Germany
Na-pyruvate	0.1mM	Gibco, Germany
L-glutamine	4mM	Gibco, Germany
Non-essential amino acids	0.1mM	Gibco, Germany
Penicillin/Streptomycin (100X)	1%	Gibco, Germany

Basal medium

Components	Concentration	Company
BME		Gibco, Germany
Fetal bovine serum	10%	Gibco, Germany
L-glutamine	1%	Sigma, Germany
D-glucose (45%)	1%	Sigma, Germany
Penicillin/Streptomycin (100X)	1%	Gibco, Germany

BME-based neuronal medium

Components	Concentration	Company
BME		Gibco, Germany
Fetal bovine serum	10%	Gibco, Germany
B-27	2%	Gibco, Germany
L-glutamine	1%	Sigma, Germany
D-glucose (45%)	1%	Sigma, Germany
Penicillin/Streptomycin (100X)	1%	Gibco, Germany

Embryonic stem cell (ES) medium

Components	Concentration	Company

DMEM, high glucose		Gibco, Germany
Fetal bovine serum	15%	Gibco, Germany
Na-pyruvate	0.1mM	Gibco, Germany
L-glutamine	4mM	Gibco, Germany
Non-essential amino acids	0.1mM	Gibco, Germany
L-alanin-L-glutamine	2mM	Gibco, Germany
β-mercaptoethanol	0.1mM	Millipore, Germany
Leukemia inhibitory factor	1000U/ml	Millipore, Germany

RPMI medium

Components	Concentration	Company
RPMI1640		Gibco, Germany
Fetal bovine serum	10%	Gibco, Germany

Macrophage GM-CSF conditioned medium

Components	Concentration	Company
Fetal bovine serum	5%	Gibco, Germany
Supernatant of 5637 cultured medium	45%	
RPMI medium	50%	

Other cell culture reagents

Opti-MEM	Gibco, Germany
Trypsin-EDTA (0.05%)	Gibco, Germany
Chicken serum	Gibco, Germany
Gelantine (0.1%)	Sigma, Germany
Poly-L-lysine	Sigma, Germany
PBS (1X)	Gibco, Germany
Tryptan blue	Gibco, Germany
Normal goat serum	Sigma, Germany
LPS	Sigma, Germany
Recombinant mouse Interferon γ	HyCult Biotechnology, Germany
Fluoresbrite Polychromatic Red Microspheres	Polysciences, Germany
TPA	Sigma, Germany
G418 (neomycin)	Sigma, Germany
M16 and M2 medium	Sigma, Germany
EndoN	AbCys S.A, France
Fluoresbrite polychromatic red 1.0 micron microspheres (the beads for phagocytosis assay)	Polysciences, USA

2.1.3 Cells and animals

Cell	Common name	Source
ES-129-MPI	MPI	kindly provided by Ahmed Mansouri, MPI Göttingen
ES-Bruce4	Bruce4	kindly provided by Frank Edenhof and Anke Leinhaas, RNB, Bonn
Murine embryonic fibroblasts (MEF)	MEF	isolated from CD1 mice (E14.5);kindly donated by Anke Leinhaas
293FT	293FT	Invitrogen, Germany
Primary murine microglia	Primary microglia	isolated from C57Bl/6J newborns (p3-5)
U937 monocytic cell	U937	ATCC, Germany
Human urinary carcinoma cell line (ATCC 5637)	5637	ATCC, Germany

Mouse strain	Source
CD1	Charles River Laboratories, Germany
C57Bl/6J	Charles River Laboratories, Germany
B6D2 F1	Charles River Laboratories, Germany

2.1.4 Antibodies

Primary antibodies

Antibody	Host	reactivity	Conjugation	Company
CD16/CD32 (FC-Block)	rat			BD pharmingen, Germany
CD45	rat		biotin	BD pharmingen, Germany
Flag	mouse			Sigma, Germany
Flag	mouse		Cy3	Sigma, Germany
IgG1κ isotype	mouse			BD pharmingen, Germany
Whole rabbit serum	rabbit			Dianova, Germany
Siglec-11	goat	human	Biotin	R&D, Germany
Iba1	rabbit	mouse		Wako, Germany
GFP	rabbit	mouse		Abcam, Germany
β III tubulin	mouse	mouse		Sigma, Germany
β- actin	mouse			Millipore, Germany

Biotin IgG1κ isotype	mouse	Biotin	BD pharmingen, Germany

Secondary antibodies for flow cytometry

Antibody	Reactivity	Host	Conjugation	Company
PE	biotin		streptavidin	Dianova, Germany
Alexa 488	rabbit	goat		Invitrogen, Germany

Secondary antibodies for immunostaining

Fluorophore	Reactivity	Host	Company
Cy3	mouse	goat	Dianova, Germany
Cy3	rabbit	goat	Dianova, Germany
Alexa 488	rabbit	goat	Invitrogen, Germany
Alexa 488	mouse	goat	Invitrogen, Germany
FITC	mouse	goat	Dianova, Germany
FITC	rabbit	goat	Dianova, German
FITC- Strepvidin	biotin		Dianova, German
Cy3- Strepvidin	biotin		Dianova, German

Secondary antibodies for Western blot

Antibody	Reactivity	Host	Conjugation	Company
streptavidin	biotin		HRP	Millipore, Germany
IgG	goat	rabbit	HRP	Dianova, Germany

Other staining reagents

DAPI	Sigma, Germany
Propidium iodide	Sigma, Germany

2.1.5 Primer (purchased from MWG, Germany)

Primers used for real-time PCR

Target	Orientation	Sequence
GAPDH	forward	5'- AACTTTGGCATTGTGGAAGG -3'
	reverse	5'- GGATGCAGGGATGATGTTCT -3'
NOS2	forward	5'- AAGCCCCGCTACTACTCCAT -3'
	reverse	5'- GCTTCAGGTTCCTGATCCAA -3'
TNF-α	forward	5'- TCTTCTCATTCCTGCTTGTGG -3'

	reverse	5’- AGGGTCTGGGCCATAGAACT -3’
IL-1β	forward	5’- CTTCCTTGTGCAAGTGTCTG -3’
	reverse	5’- CAGGTCATTCTCATCACTGTC -3’
TGF-1β	forward	5’- CAATTCCTGGCGTTACCTTG -3’
	reverse	5’- GCTGAATCGAAAGCCCTGTA -3’

Primers used for cloning

Target	Orientation	Sequence
Eco47III_XhoI_Siglec-11	forward	5’-ATATTAGCGCTCTCGAGGCCACCATGC TGCTGCTGCCCCTGCTGCT -3’
EcoRI_Siglec-11	reverse	5’-CCGGGTGAATTCTTCGAATCATCACTT TGGAACCATCCCTGACATCCCTG -3’
SfuI_4Gly_Siglec-11	reverse	5’-ATTTCGAATCCTCCTCCTCCTCCTCCCT TTGGAACCATCCCTGACATCTC -3’

Primers used for RT-PCR of Siglec-11

Primer pair	Orientation	Sequence
1	forward	5’-TCTCAGCCTCTCCGTGCACT-3’
	reverse	5’-CAAGGCAGGAACAGAAAGCG-3’
2	forward	5’-ACAGGACAGTCCTGGAAAACCT -3’
	reverse	5’-AGGCAGGAACAGAAAGCGAGCAG -3’
3	forward	5’-TGCTACCAGGGAAGCTGGAGCAT -3’
	reverse	5’-AGGCATAGTGGAGCTCCTGCTCTT -3’

Primers used for sequencing

Target	Orientation	Sequence
Siglec-11	forward	5’-CCTGAGCAATGCGTTCTTTC-3’
		5’-ACAGGACAGTCCTGGAAAACCT-3’
		5’-TGCTACCAGGGAAGCTGGAGCAT-3’
	reverse	5’-ATAAGCAGCAGTAGACTCGTCC-3’
		5’-AACACACAGATGACCGTCACCG-3’
		5’-AGGCAGGAACAGAAAGCGAGCAG-3’
		5’-AGGCATAGTGGAGCTCCTGCTCTT-3’
PLL backbone	reverse	5’-GGGTACAGTGCAGGGGAAAGAATAGTAG-3’
Iba1 promoter	forward	5’-ATCGATTACTATAGGATGCATCGTG-3’
		5’-GGGAGTTAGCAAGGGAATGAGT-3’
		5’-CAAGGCTATCCCTGGTATGAG-3’
		5’-CTCCGGGAGCTGATCTAAGTCTTTC-3’
	reverse	5’-CTCATACCAGGGATAGCCTT-3’
		5’-GTGTGGAAAGGCACCAGGAT-3’
		5’-CTGAACTTGTGGCCGTTTAC-3’

		5’-GTTTCCTGTTTGCAGGGTACAC-3’
CMV promoter	forward	5’-AAAATGTCGTAACAACTCCG-3’
PGK promoter	forward	5’-TAGCACGTCTCACTAGTCTCG-3’
	reverse	5’-GCGAAGGAGCAAAGCTGCTATT-3’
Neo	forward	5’-GGCTATGACTGGGCACAACAG-3’
		5’-GATGATCTCGTCGTGACCCATG-3’
		5’-TTTCTCGGCAGGAGCAAGGT-3’
	reverse	5’-TTTCTCGGCAGGAGCAAGGT-3’
GFP	forward	5’-ACGTAAACGGCCACAAGTTCAG-3’
		5’-ACCACTACCAGCAGAACACC-3’
	reverse	5’-TGCAGATGAACTTCAGGGTCAG-3’

2.1.6 Consumables

6-well culture plates	Cellstar, VWR International, Germany
15ml tubes	Cellstar, VWR International, Germany
50ml tubes	Sarstedt, Germany
5ml, 10ml, 25ml pipets	Sarstedt, Germany
Chamber slides	Nunc, Germany
Cryovials	VWR International, Germany
$75cm^2$, $175\ cm^2$ culture flasks	Sarstedt, Germany
5ml polystyrene round-bottom tubes	BD Falcon, Germany
3cm, 5cm, 10cm culture dishes	Sarstedt, Germany
500µl, 1000 µl plastic tube	Eppendorf, Germany
PCR tubes	Biozym Diagnostics, Germany
10µl, 100µl, 1000µl tips	Eppendorf, Germany
5ml, 10ml syringes	Braun,Germany
Needles	Braun, Germany
Glass slides for cryosectioning	Menzel, Germany
Bottle top filters (0.25µm pore)	Millipore, Germany
Filters (0.45µm, 0.2µm pore)	Sarstedt, Germany
Transwell (8µm pore filter)	Millipore, Germany

2.1.7 Equipment

Centrifuges	Sorvall Discovery 90SE, Hitachi, Germany
	Megafuge, 1.OR. Heraeus, Germany
	Biofuge Fresco, Heraeus, Germany
Cryostat	Microtom HM560, Microm Int., Germany
Flow cytometer	FACSCalibur, BD Bioscience, Germany
Electrophoresis gel chambers	Blomed Analytik GmbH, Germany

Power supply	Amersham Bioscience, Germany
Heating block	Stuart Scientific, Germany
Incubators	Heracell240, Heraeus, Germany
Laminar air flow workbench	Herasafe, Heraeus, Germany
Microscopes	Axiovert40CFL, Zeiss, Germany
	Axiovert200M, Zeiss, Germnay
	Fluoview1000 Confocal Microscope, Olympus, Germany
	Olympus SZXZ-ILLT, Olympus corporation, Japan
pH Meter	Hanna Instruments, Germany
Photometer	Eppendorf, Germany
Real time thermocycler	ABI Prism 5700 Seqeunce Detection System, Applied Biosystems, UK
Thermocycler	T3, Biometra, Germany
Vortex	$2X^2$, VelpScientifica, Germany
Transplantationsequipment	Fine Science Tools, Germany

2.1.8 Software

Openlab4.0.1	Improvision, Germany
CorelDRAW	Graphics Suite 11, Germany
EndNote X	Thomson ISI ResearchSoft, USA
Microsoft Office	Microsoft USA, USA
Olympus FluoView1.4	Olympus, Germany
SDS 2.2.2	Applied Biosystems, USA
Cellquest Pro	BD Biosciences, USA
FlowJo 6.4.7	Tree Star, USA
KaleidaGraph 4.0	Synergy, USA
SPSS 16.0	SPSS, USA
Axiovision 4.6.3	Carl Zeiss Imaging Solutions, Germany
ImageJ 1.39u	NIH, USA
CorelDRAW	Graphics Suite 12, USA
Vector NTI Advance 10	Invitrogen, USA
Gene Runner	Hasting Software, Inc. USA
Primer Premier 5.0	Premier Biosoft International, USA

2.1.9 Kits and additional reagents

RNA and DNA isolation kit

RNeasy Mini	Qiagen, Germany
RNeasy Mini for lipid tissue	Qiagen, Germany
RNAse free DNAse Kit	Qiagen, Germany
QIAprep Plasmid Miniprep	Qiagen, Germany
Endofree Plasmid Maxiprep	Qiagen, Germany
Min iElute Gel extraction	Qiagen, Germany
QIAquick Gel extraction	Qiagen, Germany
Red Extract-N-Amp Tissue PCR Kit	Sigma, Germany

Additional reagents

Lipofectamine2000 reagent	Invitrogen, Germany
Ampicilin	Sigma, Germany
LB agar and LB media	Fluke Biochemika
DMSO	Sigma, Germany
Propidium Iodide	Sigma, Germany
Glycerol	Sigma, Germany
Tissue tek O.C.T. compound	Sciences Services, Germany
Ethanol	Sigma, Germany
Vectashield	Vector Laboratories, USA
Bovine serum albumin	Sigma, Germany
Hexamer random primers	Roche, Germany
Trizol Reagent	Invitrogen, Germany

2.1.10 Anesthethics

Animals were anesthetized with Ketamin intraperitoneal (i.p.) (Ketamin 100 mg per kg body weight) before embryo transplantation or perfusion.

2.2 RT-PCR analysis of Siglec-11

RNA was isolated from human brain tissue derived from patients undergoing epilepsy surgery (kindly provided by the Department of Neurosurgery and Epileptology of the University Hospital Bonn) using RNeasy Mini for lipid tissue Kit or from cultured cells

using Trizol Reagent. Reverse transcription of RNA was performed with SuperScript III reverse transcriptase and hexamer random primers using 5µg total mRNA. Primer pairs designed according to the coding region of Siglec-11 gene were used to detect the expression of Siglec-11. PCR was amplified for 40 cycles. Amplified product was electrophoresed on 1% agrose gel.

2.3 Plasmids construction

PLL3.7 (provided by L. van Parijs, MIT, Cambridge, MA) was modified to contain a neomycin selection marker by replacing the U6 promoter with a cassette of phosphoglycerate-kinase (PGK) promoter and neomycin resistant gene. Plasmids expressing GFP (Invitrogen), Siglec-11 (RZPD, Deutsches Ressourcenzentrum fuer Genomforschung GmbH), flag and GFP tagged Siglec-11 were cloned based on the modified pLL3.7 back bone behind a cytomegalovirus (CMV) promoter. In some cases the CMV promoter was replaced by ionized calcium-binding adaptor molecule 1 (Iba1) promoter. Described genes or fragment were obtained from corresponding constructs by PCR using extended primers allowing the product to be inserted into the following vectors by specific restriction sites. Digested vector backbone and insert were ligated and transformed to Top10 competent cells. Positive colonies selected by antibiotics were inoculated in a small volume. Plasmid DNA was isolated and restrictively digested using the corresponding enzymes. Colonies having the insert were expanded and purified using EndoFree Maxi Kit (Qiagen,Germany). The sequence of each plasmid was verified further by sequencing.

2.4 Viral particle production

The 293FT (purchased from Invitrogen and expanded in the laboratory) packaging cell line was kept in Dulbecco's Modified Eagle's Medium (DMEM; Gibco) with 1% penicillin/streptomycin and glucose at 37°C in 10% CO2. Viral particles were produced in

10 cm dishes precoated with poly-L-Lysine. Three µg of targeting plasmid together with 3µg of each of the three packaging helper plasmids were co-transfected using Lipofectamine 2000 reagent (Invitrogen) to 6 million 293FT cells, which were expanded at least once before transfection. Cells were transfected in Opti-MEM medium without antibiotics containing 10% FBS. Medium was replaced by fresh DMEM 6 to 16 hours post-transfection. Viral supernatant (10 ml) was collected at 48-72 hrs post-transfection. Viral particles were then ultracentrifuged using a Sorvall DiscoveryTM 90SE ultracentrifuge at 25000 g (19600 rpm) for 90 min at 4°C to increase titers. Concentrated viral particle pellet were resuspended in 300 µl PBS by slightly shivering overnight at 4°C. Viral particles were either immediately applied to transduce cells or stored at -80°C in small aliquots for further usage.

2.5 Lentivial transduction of cells

To transduce adherent cells, cells were normally seeded at a density of $5x10^5$cells/ml in 6-well dish and incubated overnight to 24 hours with 10^8 Transducing Units (TU)/ml of lentiviral particles (50 µl viral particle per ml medium).

To transduce cells in suspension, viral particles were added to $5x10^5$cells/ml cells in 6-well dish and first centrifuge at 2400 rpm, 32°C for 90 min. After centrifugation step, cells were kept in 37°C and 5% CO2 incubator. 24 hours post-transduction, medium were refreshed into complete culture medium. In cases to enrich the positively transduced cells, neomycin (also know as G418) selection was applied at a concentration of 400 to 1000 µg/ml (according to the cell killing curve of different cell types) from 48 hours post-transduction for 5 days.

To transduce mouse ES cells, cells were plated at a density of 3 $x10^5$cells/well in 6-well dish with MEF feeders. Six hours later, when the ES cells were attached but still in single-cell condition, the medium was refreshed with 50 µl viral particle in 1 ml medium and incubate for 24 hours. Medium was changed afterwards and cells were spilt into 10 cm dish 48 hours post-transduction. G418 was applied then as to the other cell types for 10 days at a concentration of 230 µg/ml. Single ES cell colony surviving the selection

were picked out manually to 96-well plate with MEF feeder cells. After verifying the incorporation of the target gene by PCR, the positively transduced colonies were expanded and used for further experiments.

2.6 PCR analysis of the incorporation of the lentiviral vector to the transduced cells

Genomic DNA was isolated from the cells using QIAmp DNA Mini Kit. GAPDH was used as a positive control for the quality and quantity of DNA. Primers specific for Siglec-11 gene were used to detect the incorporation of the gene to the genome of the target cells. PCR was amplified for 40 cycles. Amplified DNA was electrophoresed on 1% agrose gel.

2.7 Culture of primary microglia

Primary microglia were prepared from brains of postnatal day 3 or 4 (P3 or P4) of C57BL/6 mice. In brief, meninges were removed mechanically. Cells from hippocampus and cortex were isolated and dissociated by triturating and cultured in basal medium for 14 days to form a confluent mixed glial monolayer. To collect microglial cells, the cultures were shaken on a rotary shaker (350 rpm) for 3 hours. The detached microglial cells were seeded on PLL coated culture dishes. Purity of the isolated microglia was determined by flow cytometry analysis with antibody directed against CD11b.

2.8 Culture of 5637 cell line (human urinary bladder carcinoma)

5637 is a growth factor producing cell line established from a human primary bladder carcinoma. The supernatant of the cell line was used as a substitute for the rhGM-CSF. The cells were cultured in tissue culture flasks in RPMI medium at 37°C with 5% CO_2. Confluent cells were split in a 1:5 ratio. The supernatant of the cell line was collected

every 3rd days. Dead cells and cell debris were removed from the supernatant by centrifuging for 10 minutes at 1000rpm. The supernatant was then passed through a 0.2µm filter and used as a substitute for rhGM-CSF to prepare macrophage GM-CSF conditioned medium.

2.9 Culture and differentiation of human monocytic cell line

The human monocytic cell lines U937 (ATCC, Germany) was maintained in RPMI medium supplemented with 10% FBS. New cultures of $2x10^5$/ml were made when the cell density was about $10x\ 10^5$/ml. To differentiate the suspending cells into macrophages, cells were treated with 200 ng/ml of TPA (Sigma, Germany) in macrophage GM-CSF conditioned medium for 24 hours. Attached cells were further maintained in macrophage GM-CSF conditioned medium. Trypsin was applied to detach the cells from the dish when necessary.

2.10 Culture of ES cells

One or two days before starting the culture of ES cells, irradiated MEF feeders were prepared in tissue culture plates pre-coated with gelatin (0.1%) at a density of $0.5x\ 10^5$ cells/cm^2 to form a monolayer. ES cells were seeded to the feeder cultures at a density of $3\ x\ 10^6$ cells / 100 mm plate in ES culture medium, kept at 37°C with 5% CO_2. The cells were examined and media was changed daily. To split the cells, 0.25% trypsin-EDTA was used for MPI ES cells (MPI, Göttingen), while 2% of chicken serum was added to the 0.25% trypsin-EDTA when splitting the Bruce4 ES cells (Frank Edenhof and Anke Leinhaas, Bonn).

2.11 Immunocytochemistry of cultured cells

Cells were fixed in 4% PFA for 15 min at room temperature (RT), blocked by 5% bovine

serum albumin (BSA), 5% normal goat serum (nGS) and 0.1% Triton-X for 30 min, and then immunostained with a primary antibody diluted in the blocking solution at the right concentration for overnight at 4°C or for 1 hour at RT. A chromophore-conjugated secondary antibody was prepared in blocking solution and applied to the cells at RT for 1 hour. Double-labeling was performed by mixing the primary antibodies from different producer species followed by chromophore-conjugated secondary antibodies that could avoid the overlap of emission length. For example Cy3 (3 μg/ml) /Alexa488 (2.5 μg/ml) combination were used. Nuclei of immunostained cells were subsequently labeled with 4', 6-diamidino-2-phenylindole (DAPI) (0.1 μg/ml). Images were collected by confocal laser scanning microscopy (Fluoview 1000, Olympus) or fluorescence microscopy (Axioskop2, Zeiss).

2.12 Analysis of cytokine gene transcripts by real-time RT-PCR

RNA was isolated with the RNeasy Mini Kit from 0.5 x 10^5 primary microglia after stimulation with 500ng/ml LPS for 24 hours. Reverse transcription of RNA was performed with SuperScript III reverse transcriptase and hexamer random primers. Quantitative RT-PCR with specific oligonucleotides was performed with SYBR Green PCR Master Mix using the ABI 5700 Sequence Detection System and amplification protocol for the ABI 5700 Sequence Detection System. Amplification specificity was confirmed by the analysis of the melting curves. Results were analyzed with the ABI 5700 Sequence Detection System v.1.3 after establishing the reaction efficiency for each primer pair. Quantification using the delta-CT method was carried out.

2.13 Western blot analysis of the protein expression

Total protein was isolated from human brain tissue using Trizol Reagent, followed by chloroform，ethanol and isopropanol precipitation. Briefly, the tissue was homogenized in Trizol Reagent at 1ml per 1mg tissue. Chloroform was added to the homogenate to separate it into aqueous and organic phases after centrifugation. The interphase and the

organic phase contained DNA and proteins. Sequentially DNA was precipitated with ethanol. Proteins were isolated from the phenol-ethanol supernatant obtained after precipitation of DNA and washed with 3 times of 0.5 ml of 0.3 M guanidine hydrochloride in 95% ethanol and one time of 100% ethanol. The protein pellet was then resuspended in 1% SDS and stored at -80 ^{0}C. To prepare protein lysate from cultured cells, cells in culture dish were rinsed once with PBS at RT. RIPA buffer (Sigma, 10^7cells/ml) was added to homogenize the cells on ice. Adherent cells were further removed with a cell scraper. The resulting lysate was transfered to a microcentrifuge tube and gently rocked at 4° C for 15 minutes. Afterwards, cell lysate was centrifuged at 10,000 g for 10 minutes at 4° C. The supernatant was collected to a new microcentrifuge tube as the whole cell lysate. Protein concentration was measured using the BCA Kit from Thermo Scientific Pierce Protein Research Products. 50 µg of protein was loaded to 10% NuPAGE® Novex Bis-Tris Gels. SDS-PAGE was carried out using the NuPAGE® Electrophoresis System according to the manufacture. Biotinylated anti-Siglec-11 antibody and anti-β-actin antibody was used at 0.2 ug/ml and followed by proper secondary antibody to blot the target proteins. Antibody binding was visualized with SuperSignal West Pico Chemiluminescent Substrate Kit (Pierce Biotechnology).

2.14 Removal of PSA by EndoN treatment

EndoN (AbCys S.A, France) was diluted in the medium of the culture at a concentration of 0.5 U/ml and added to the cells. Cells were incubated at 37^{o}C for at least 5 hours.

2.15 Microglia-neuron co-culture

Primary neuronal cultures were prepared from hippocampus and cortex of C57BL/6 mice embryos (E15) as described previously (Neumann H. et al, 2002). Briefly, neurons were isolated from whole brains of embryonic day 16 mice, and the meninges were removed. Cells (5 x 10^3/ml) were plated into dishes that had been pre-treated with poly-L-ornithine (0.5 mg/ml; Sigma, St. Louis, MO) and were cultured in BME-based neuronal medium.

Neurons at day 4 post-preparation were used to co-culture with micrglial cells. Microglia were added to the neuron culture at the ration of 1:10 (micorglia to neuron) in the BME-based neuronal medium. 48 hours later, cells were fixed with 4% PFA at RT for 15 minutes.

2.16 Neurite and neuroal cell body evaluation

Immunocytochemistry was performed after fixation with 4% PFA. Cells were stained with β-tubulin-III antibody followed with Cy3 conjugated secondary antibody and subsequently DAPI labeling of the neulei. Five randomly selected areas in each dish were scanned and analyzed by confocal microscopy. β-tubulin-III positive neuritis, which crossed two of four 500-μm-long parallel lines (distance of 100 μm), were counted. Total number of nuclei stained with DAPI and double labeled with antibodies directed against β-tubulin-III was counted in five microscopic fields. Value of the co-cultures with Siglec-11 expressing microglia was normalized to that of the the co-cultures with control vector expressing microglia.

2.17 Aβ phagocytosis assay

Biotinylated Aβ 42 peptide was kept at RT to allow the formation of aggregates. To analyze the phagocytosis capacity, cells were treated with Aβ (10 μg/ml) for 1.5 hour. Cells were fixed in 4% PFA and then permeabilized with 0.1% Triton X-100. Fixed cells were stained with Cy3-conjugated streptavidin. Microglial cells were visualized by staining of CD45 antibody followed with the FITC conjugated secondary antibody. Analysis was made using fluorescence microscopy. 5 photographs were taken of each well and cells emitting a yellow signal were classified as phagocytosing and counted accordingly.

2.18 Generation of transgenic mice from embryo-stem cell aggregation

CD-1 mice were used as embryo donors. 4-week old CD-1 femals (Charles River) were superovulated by administering intraperitoneally pregnant mare's serum (PMS), which is used to mimic follicle-stimulating hormone (FSH), and 47 hours later human chorionic gonadotropin (hCG), which is used to mimic luteinizing hormone (LH), each at a dose of 5 IU/mice. The females were then mated with stud males. Next, females with a copulation plug were picked out for embryo collecting. 8-cell stage embryos were collected from 2.5 days post-coitum (dpc) females. The zona pellucida of the embryos was removed using an acidic tyrode solution. Small drops (50 μl) of M16 medium (Sigma) were placed on the bottom of a 60-mm sterile plastic culture dish. Six or more depressions in each microdrop were punched with aggregation needles. Zona pellucida free embryos were washed 3 times in M2 medium and transferred into these microdrops, one embryo inside each depression. ES cells lentiviral transduced with Siglec-11 and positively picked by PCR analysis were trypsinized for short time (1 minute) to detach from the culture dish but avoid dissociating them into single cells. ES cell clumps formed after incubating in M2 medium on ice for 20 minutes. Clumps with around 16 ES cells in each were placed on the top of the embryo in a depression in the microdrop. The aggregates were culture overnight at 37^{o} C, 5% CO2. The next day, 10-15 blastocyts and/or compact morulas were transferred into one uterine horn of each 2.5 dpc pseudopregnant recipient CD-1 mouse. Chimerical mice with 30% or more ES cell derived coat color were breed with CD-1 mice at age of 6 weeks to check for germ-line transmission.

2.19 Generation of transgenic mice from pronuclear injection

The pronuclear injection experiments were carried out in HET (Haus für Experimentelle Therapie) by the facility. For the pronuclear injection, the Iba1 promoter, cDNA of Siglec-11, together with WPRE cassette were separated from the vector by NotI and BsiEI digestion followed by 1% agrose gel electrophoresis. DNA fragment was isolated from

gel using the QIAquick Gel extraction kit (Qiagen) according to the manufacture's instructions and supplied to the facility upon request. Otherwise, DNA was stored at a concentration of about 100ng/ul at -80 ^{o} C. 3-5 ng/µl of DNA was used to inject into E0.5 zygotes collected from superovulated femal zygotes donors and transferred to pseudopregnant recipient CD-1 mouse according to established procedures. Newborns were genotyped at age of 2 to 4-week by PCR of DNA from the tail tip using primer pair 3 of Siglec-11. Founders with the transgene were further breed to establish germ-line transmitting lines.

2.20 Genotyping of mice

Mouse tail tips were cut at the age of 2 to 4 weeks. DNA was prepared using the Red Extract-N-Amp Tissue PCR kit (Sigma) according to the manufacture's instruction. PCR reaction was carried out using the Siglec-11 specific primers with 40 cycles. Amplified DNA was electrophoresed on 1% agrose gel.

2.21 Statistics

Data are presented as mean ± SEM of at least 3 independent experiments. Data were analyzed by ANOVA using SPSS computer software.

3 RESULTS

3.1 Detection of splice variant 2 of Siglec-11 in human brain tissue

In order to confirm the expression of Siglec-11 in human microglia, we analyzed human brain tissue by RT-PCR. In literature, it is described that Siglec-11 consists of 11 exons separated by introns. Three distinct primer pairs derived from different exons of Siglec-11 gene were designed (Table 3-1) to check the transcription of Siglec-11. Human brain tissue was kindly provided by the Department of Neurosurgery and Epileptology of the University Hospital Bonn and was derived from patients undergoing epilepsy surgery. Total RNA was isolated freshly from three human brain tissue samples. Reverse transcription was carried out immediately afterwards to avoid any degradation of RNA. Siglec-11 was detected in all the samples tested (Figure 3-1). However, the detected gene product was 288 bp shorter than the product that was firstly described in literature. Further analysis showed that the amplified gene product was derived from the second splice variant of Siglec-11 (Clark, H.F. et. al., 2003). This variant 2 of Siglec-11 lacks one exon (exon 8 as indicated in Figure 3-1), which is coding one out of the five Ig-like domains (the Ig-like domain 5) in the extracellular part.

Table 3-1. Primers used for RT-PCR analysis of Siglec-11

Primer pair	Orientation	Product size (bp)
1	Forward: 5'-TCTCAGCCTCTCCGTGCACT-3'	103
	Reverse: 5'-CAAGGCAGGAACAGAAAGCG-3'	
2	Forward: 5'-ACAGGACAGTCCTGGAAAACCT -3'	352
	Reverse: 5'-AGGCAGGAACAGAAAGCGAGCAG -3'	
3	Forward: 5'-TGCTACCAGGGAAGCTGGAGCAT -3'	294
	Reverse: 5'-AGGCATAGTGGAGCTCCTGCTCTT -3'	

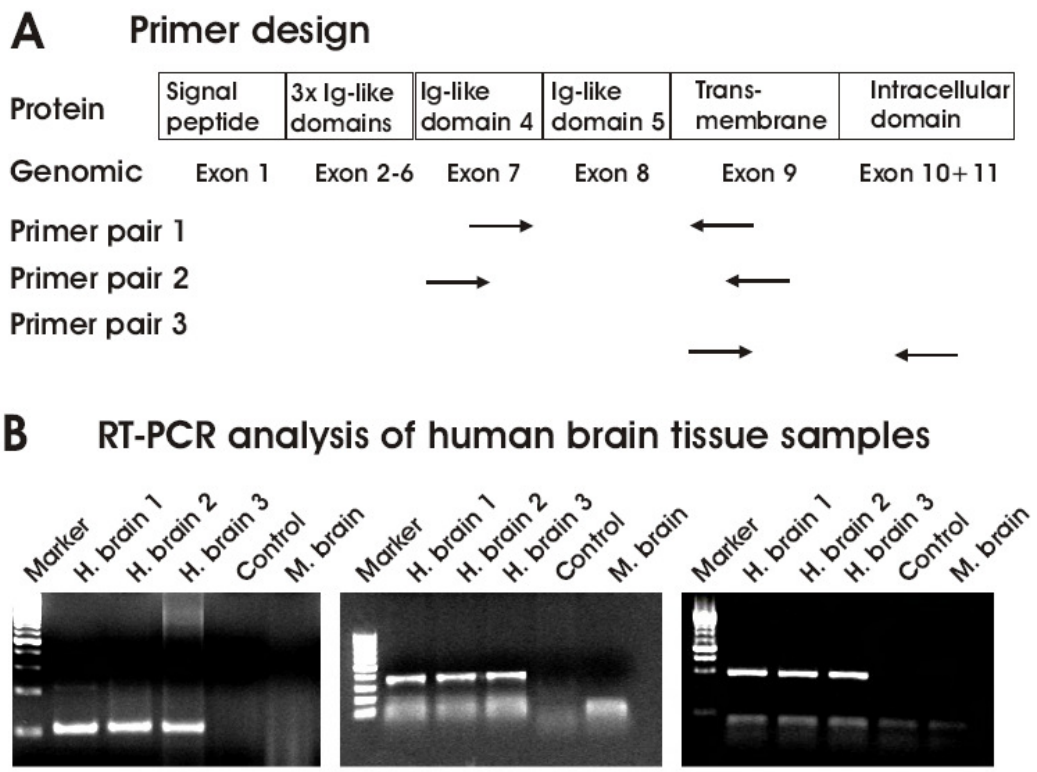


Figure 3-1. Detection of splice variant 2 of Siglec-11 in human brain tissue by RT-PCR. A: cDNA and protein structure of Siglec-11 as first reported by Angata T., et.al. The arrows show the sites of the forward and reverse primers used to detect the Siglec-11 gene expression corresponding to the protein and cDNA. Three different human brain tissues were checked. RNA from the human brain tissue and cDNA from mouse brain tissue were used as negative control.

To confirm the expression of Siglec-11 at protein level, Western blot analysis was carried out. Total protein isolated from three human brain tissue samples was analyzed. Protein lysate from 293 cells lentivirally transduced with or without the splice variant 2 of Siglec-11 was used as control. Results showed that Siglec-11 expression was detected in all the three samples (Figure 3-2). Furthermore, the protein size of the human brain samples was the same compared to the one in 293 cells, indicating that in these human brain samples the variant 2 of Siglec-11 was predominately expressed.

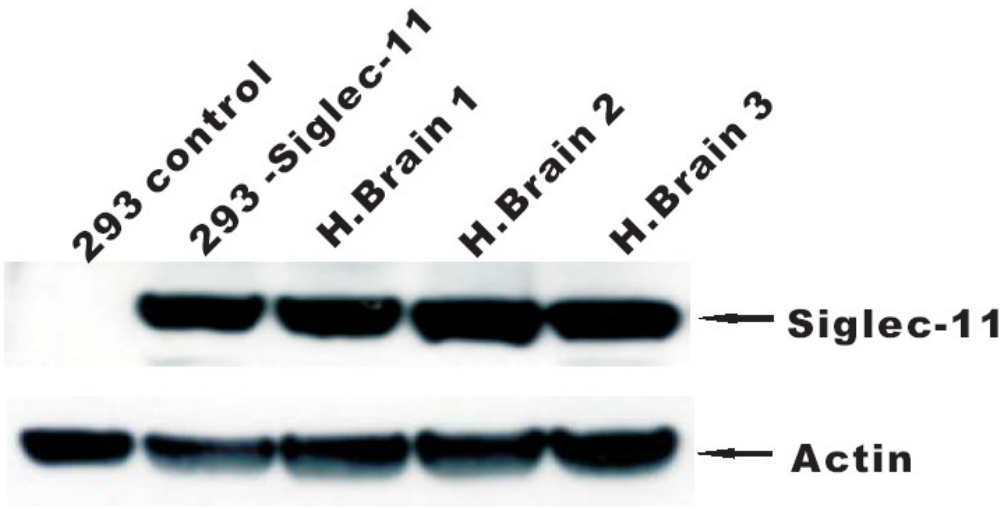


Figure 3-2. Detection of splice variant 2 of Siglice-11 in human brain tissue by Western blot. Three different human brain tissues were analyzed. Protein lysates from 293 cells transduced with or without Siglec-11 were used as negative and positive control.

3.2 Induction of Siglec-11 in macrophages differentiated from human monocytes

It was reported that Siglec-11 was detected in human tissue macrophages but not in peripheral blood cells. Monocytes in the blood circulation have the ability to differentiate into tissue macrophages. Therefore, we checked whether Siglec-11 can be induced in macrophages differentiated from monocytes. The human monocytic cell line U937 was differentiated into macrophages by stimulation with 200 ng/ml TPA (12-O-tetradecanoylphorbol-13-acetate) for 24 hours. Afterwards, the differentiated cells were treated with either LPS or INF-γ for 48 hours and RNA was subsequently isolated. RT-PCR showed that Siglec-11 was not detected in undifferentiated monocytes, but was detectable in the differentiated macrophages (Figure 3-3). However, the expression levels of Siglec-11 were quite low and undetectable by flow cytometry analysis (data not shown).

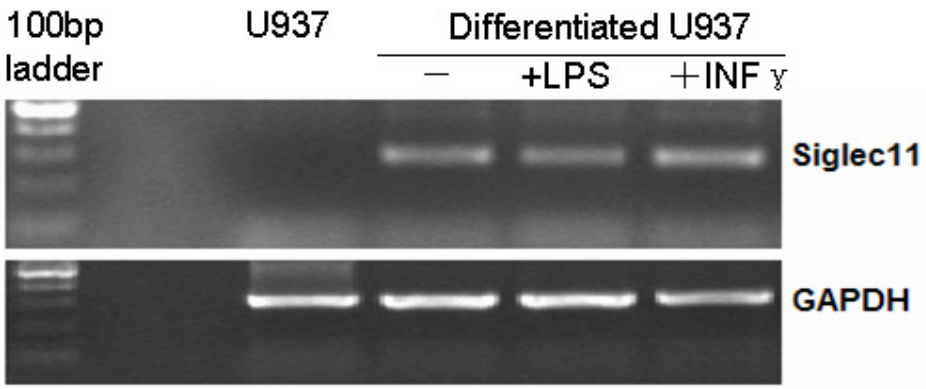


Figure 3-3. Induction of Siglec-11 in macrophages. The human monocytic cell line U937 was differentiated into macrophages and analyzed by RT-PCR.

3.3 Lentiviral expression of Siglec-11

3.3.1. Molecular cloning of the lentiviral vectors

Siglec-11 was tagged with three time flag at the C-terminal and GFP at the N-terminal which was shortly termed as fSiglec-11-GFP. In detail, the CMV-3xflag cassette was obtained from pReceiver-M12a (RZPD, Germany) by PCR which added the restriction sites of NotI and EcoR47III to replace the CMV promoter in the PLL3.7 vector by subcloning. Then, the Siglec-11 gene lacking the stop codon was subcloned in front of the GFP using the SfuI and EcoR47III restriction sites. Four times of GGA bases were added by PCR to make a 4-time glycine linker between the Siglec-11 and GFP protein.

Siglec-11 tagged with or without GFP under the control of CMV promoter (shortened as CMV-Siglec-11-GFP and CMV-Siglec-11) was modified on the basis of fSiglec-11-GFP by removing the flag tag and GFP tag in subcloning.

Siglec-11 tagged with or without GFP was also cloned to the PLL3.7 lentiviral vectors under the control of Iba1 promoter using similar strategies.

All these vectors were also having a PGK-Neomycin selection marker in front of the Sigelc11 related expression cassette. All transgenes and their associated promoters were flanked by two loxP sites (Figure 3-4).

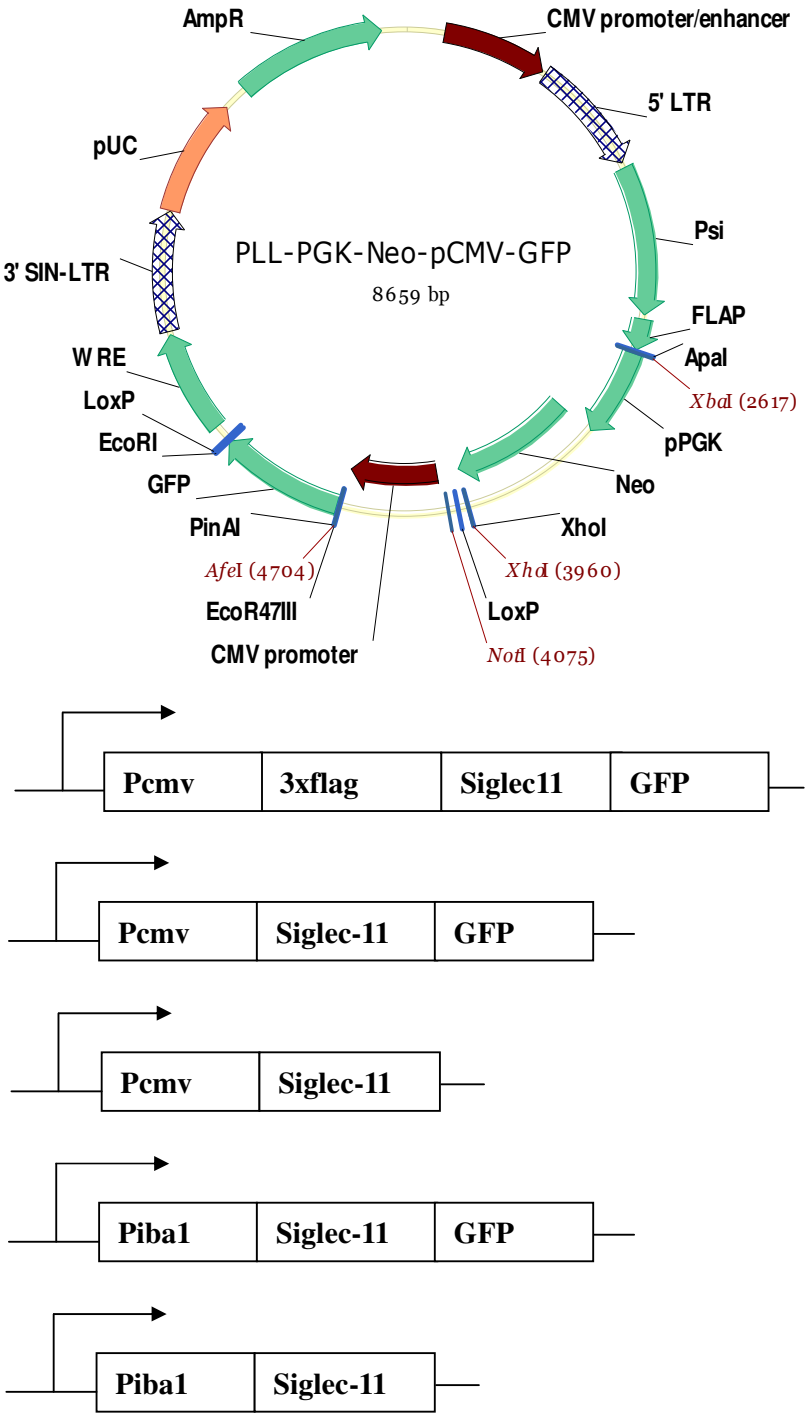


Figure 3-4. Sketch map of the lentiviral vectors. Top: schematic map of PLL-PGK-Neo-CMV-GFP. Bottom: all cloned lentivral vectors to over-express Siglec-11 tagged or non-tagged with flag and/or GFP under different promoters in the backbone of PLL-PGK-Neo-CMV-GFP.

3.3.2 Verification of Siglec-11 expression in 293 cells

The efficiency of the vectors was tested in 293 cells except the vectors in which the transgene was under the Iba1 promoter, which is known as a microglial specific promoter. 293 cells were lentivirally transduced with the corresponding vectors, and stained with anti-Siglec-11 antibody followed by flow cytometry analysis. Results showed that the transduced 293 cells were positively stained indicating that all three vector variants (fSiglec-11-GFP, Siglec-11-GFP and Siglec-11) led to an expression of Siglec-11 on the cell surface (Figure 3-5). However, the efficiency of the vectors differed. Transduction of 293 cells with fSiglec-11-GFP (17.3%) was less effective compared to Siglec-11-GFP (98.3%) and Siglec-11 (97.5%).

Transduced 293 cells were further analyzed by immunofluorescent staining with anti-Siglec-11 antibody. Siglec-11 was detected on the cell surface of 293 cells after lentiviral transduction (Figure 3-6).

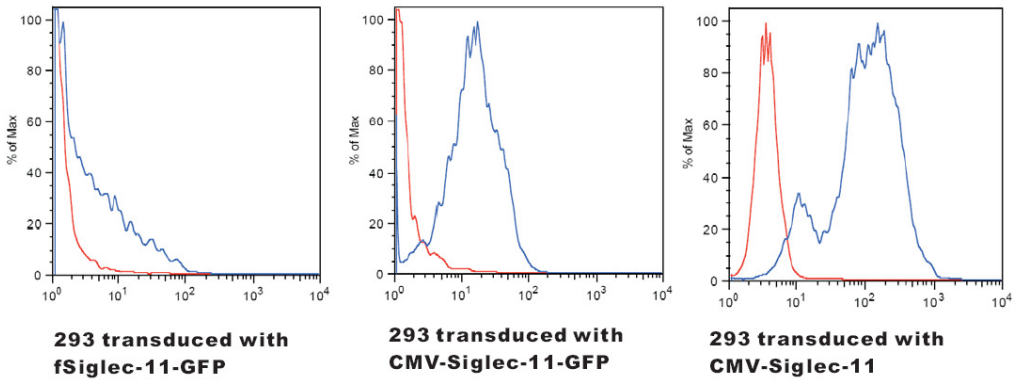


Figure 3-5. Verification of Siglec-11 expression in 293 cells. Representative flow cytometry results showed that Siglec-11 was expressed in 293 cells after lentiviral transduction.

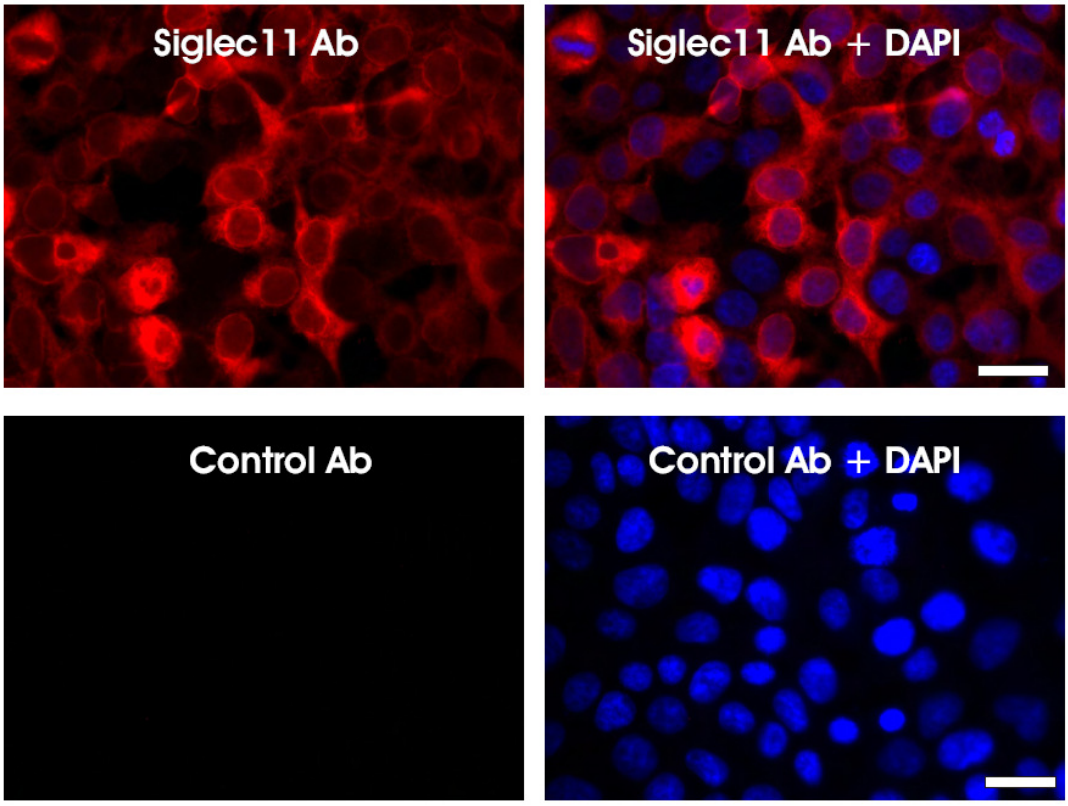


Figure 3-6. Immunocytochemistry of 293 cells after lentiviral transduction with Siglec-11. Cells were transduced with CMV-Siglec-11 vector and stained with biotinylated anti-Siglec-11 antibody followed by Cy3 conjugated secondary antibody. Scale bar: 50 µm.

3.3.3 Transduction of primary mouse microglia

Primary mouse microglia were isolated from glial cultures which were derived from neonatal mice. Cells were transduced with lentiviral vectors by centrifugation for 90 minutes. The expression of Siglec-11 was verified by RT-PCR using the Siglec-11 specific primer pair 3 (Figure 3-7).

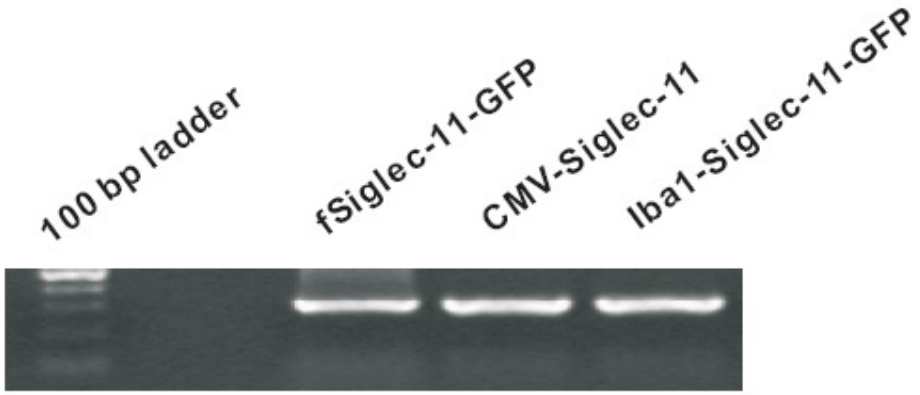


Figure 3-7. **Verification of the expression of Siglec-11 in mouse primary microglia**. Primary mouse microglia were transduced with fSiglec-11-GFP, CMV-Siglec-11 and Iba1-Siglec-11-GFP. RT-PCR was carried out using the Siglec-11 specific primer pair 3.

3.4. Functional analysis of Siglec-11 in primary microglia

3.4.1 Cytokines profile after antibody cross-linking of fSiglec-11 in primary microglia

Primary microglia were isolated from mixed glial culture and transduced with lentiviral particles to overexpress fSiglec-11-GFP or GFP. Afterwards, cells were stimulated with flag antibody or isotype control antibody for 48 hours in combination with or without LPS treatment. Cytokine profile was analyzed by real-time PCR. Values collected from cells after LPS stimulation were normalized to those of without LPS stimulation. Cross-linking with the flag antibody inhibited the expression of IL-1β and NOS2 in Siglec-11 transduced primary microglial cells (1.81±0.11 and 1.21±0.59, n=4) when compared to the control vector transduced cells (28.375±3.53 and 7.06±2.39, n=4). While there was no significant difference observed on the relative expression level of TGF-β1 and TNF-α (0.69±0.07 and 1.22±0.75 in fSiglec-11-GFP cells versus 1.03±0.15 and 3.85±0.88 in GFP cells, n=4) (Figure 3-8).

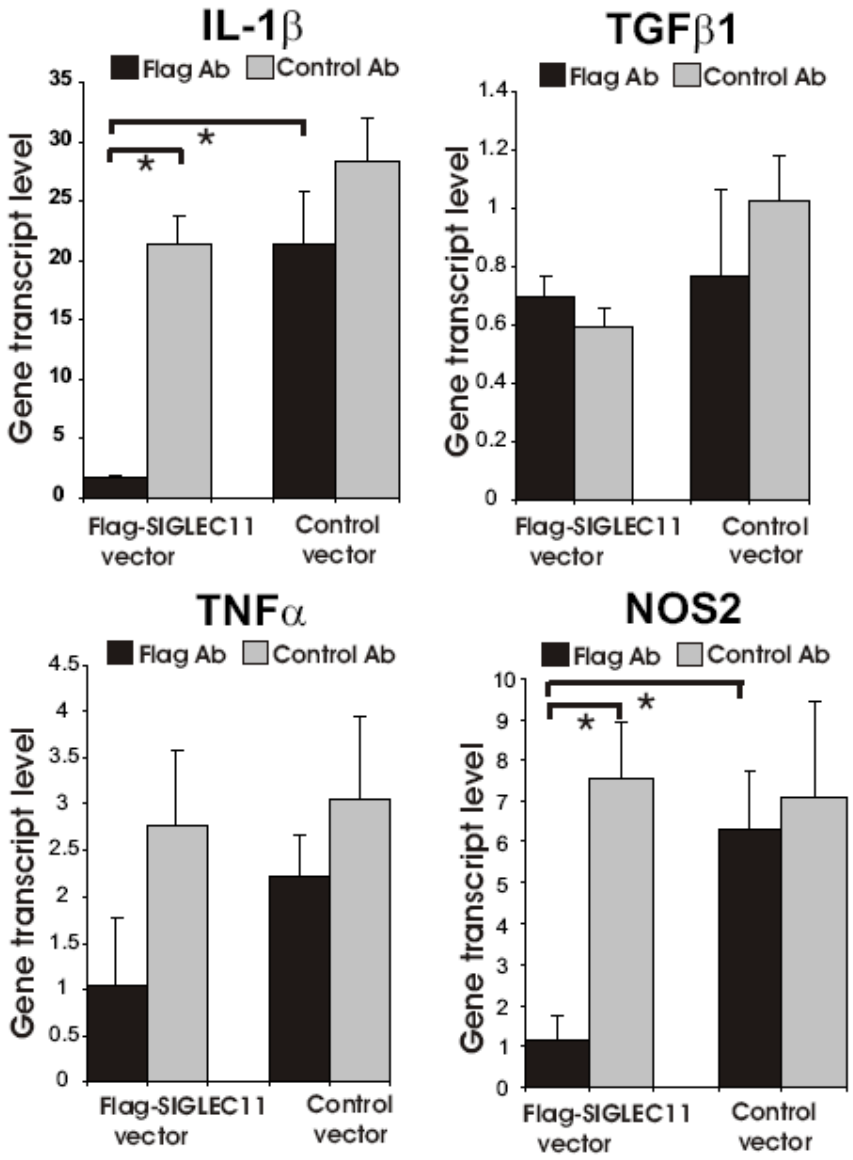


Figure 3-8. Cytokine profile after antibody cross-linking of fSiglec-11 in primary microglia. Primary murine microglial cells were lentivirally transduced with fSiglec-11-GFP vector or GFP vector. Transduced cells were cultured on plates pre-coated with antibodies directed against the flag-tag or a control antibody and stimulated with or without 500 ng/ml LPS. Gene transcripts of microglial cells were studied after 48 hours of culture by real-time RT-PCR. The values of the LPS stimulated cells were normalized to those of the cells without LPS stimulation. Data are shown as means +/- SEM, n=4. * $P < 0.05$.

3.4.2 Aβ phagocytosis assay of Siglec-11 expressing primary microglia

To investigate whether Siglec-11 has a role in Aβ phagocytosis, primary microglia were

transduced with Siglec-11 or the control vector. 48 hours post-transduction, Aβ was added to the cells for 1 hour. Cells were then fixed and visualized by staining of CD45-FITC and Aβ-Cy3. The percentage of phagocytosing cells out of the total cells captured in one visual field was calculated. Data showed that Siglec-11 transduced cells had a significant lower ratio of phagocytosing cells (44.17±10.1%) when compared to control cells which had 60.77±5.4% of the cells phagocytosing (Figure 3-9).

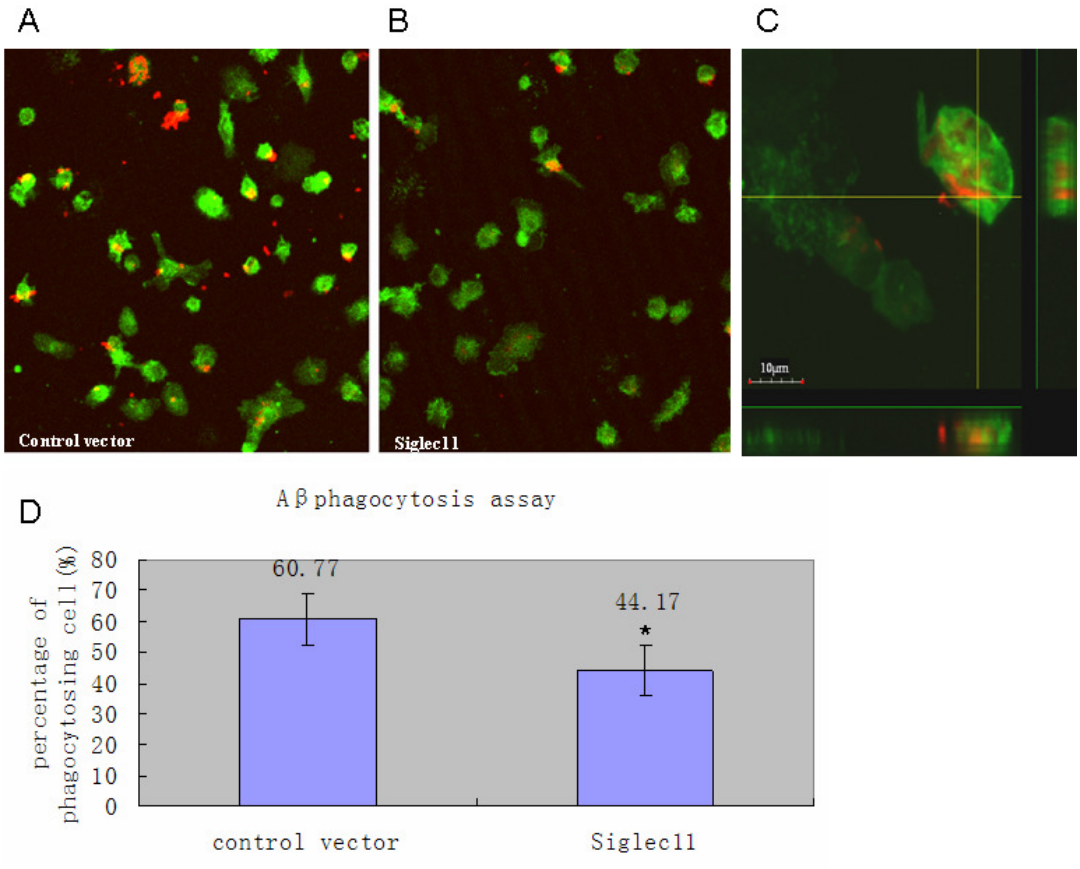


Figure 3-9. Aβ phagocytosis assay of primary microglia transduced with Siglec-11 or control vector. A. Siglec-11 transduced cells. B. Control vector transduced cells. C. A representative confocal picture showing microglial cells phagocytosing Aβ peptides. D. Statistical analysis showed less Aβ phagocytose in Siglec-11 transduced microglia compared to control vector transduced cells. * $P < 0.05$, n=4.

3.4.3. Co-culture of Siglec-11 transduced microglia and primary neurons

Microglia are known for their ability to control the death and synaptic properties of neurons (Alain Bessis, 2007). To investigate whether Siglec-11 plays a role in

neuron-microglia interactions, microglia were transduced with Siglec-11 vector and co-cultured with neurons. It has been shown that Siglec-11 binds weakly but specifically to α-2–8-linked sialic acids (NeuAc-alpha 2-8) (Angata, 2002). While the natural ligand of Siglec-11 is unknown yet, polysialic acid (PSA), which is present prominently in the nervous system, emerges as a possible ligand candidate for Siglec-11. PSA is the linear homopolymers of α-2, 8-linked Nacetylneuraminic acid (NeuAc-alpha 2-8)n, with n more than 10. In mammalian cells, most PSA is associated with neural cell adhesion molecule (NCAM). Expression of PSA-NCAM is abundant in embryonic nervous system. Using a monoclonal antibody specific to PSA-NCAM, we identified the expression of PSA not only in embryonic neurons (Figure 3-10), but also in neonatal microglia (Figure 3-11). To investigate whether Siglec-11 functions through PSA, EndoN was applied to remove PSA from NCAM in the cultures. The density of neurite and neuronal cell bodies was measured after co-culturing neurons and microglia transduced with Siglec-11 or control vector. It showed that when PSA was present in the neurons, co-cultures with Siglec-11 expressing microglia had relatively higher neurite density and neuronal cell body density compared to the control co-cultures where microglia expressed control vector (Figure 3-12). Particularly, the relative neurite density of co-cultures with Siglec-11 expressing microliga compared to those of control co-cultures was 1.6±0.2 times (mean±SEM) and 1.8±0.2 times (mean±SEM) respectively when microglial PSA was or was not present. And the relative neuronal cell body density of co-cultures with Siglec-11 expressing microglia compared to those of control cultures was 2.1±0.4 times (mean±SEM) and 2.1±0.3 times (mean±SEM) respectively when microglial PSA was or was not present. However, when PSA was removed from the neurons, the difference in the density of neurons was not observed anymore. Particularly, the relative neurite density of co-cultures with Siglec-11 expressing microliga compared to those of control cultures was 1.1±0.2 times (mean±SEM) and 1.1±0.3 times (mean±SEM) respectively when microglial PSA was or was not present. And the relative neuronal cell body density compared to those of control cultures was 1.3±0.3 times (mean±SEM) and 1.0±0.2 times (mean±SEM) when microglial PSA was or was not present. This indicated that PSA expressed on the neurons but not on microlia might contribute to the neuronal protective function of Siglec-11.

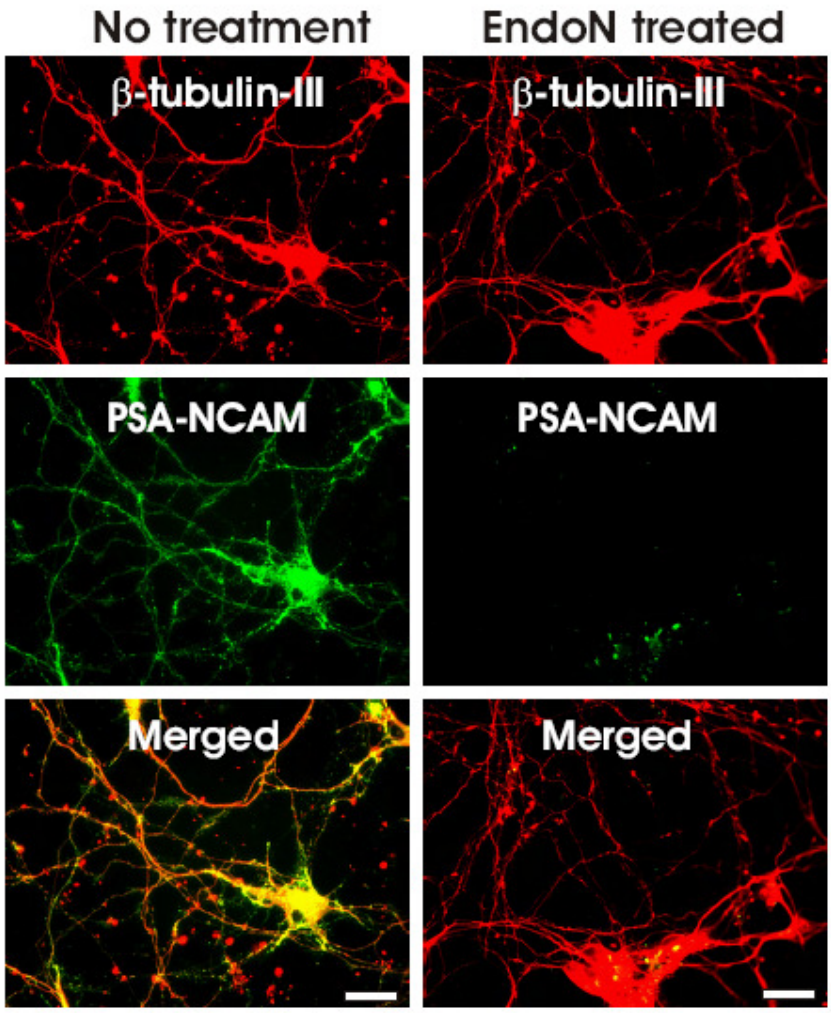


Figure 3-10. Immunostaining of PSA-NCAM on cultured primary neurons. The mouse monoclonal anti-PSA-NCAM antibody and FITC conjugated secondary antibody were used to stain PSA-NCAM on embryonic neurons. Neurons were doubled stained with anti-β-tubulin III antibody and Cy3 conjugated secondary antibody. One the right panel, EndoN was applied the culture for 5 hours, which removed the PSA-NCAM from the neurons. Scale bar: 50 μm.

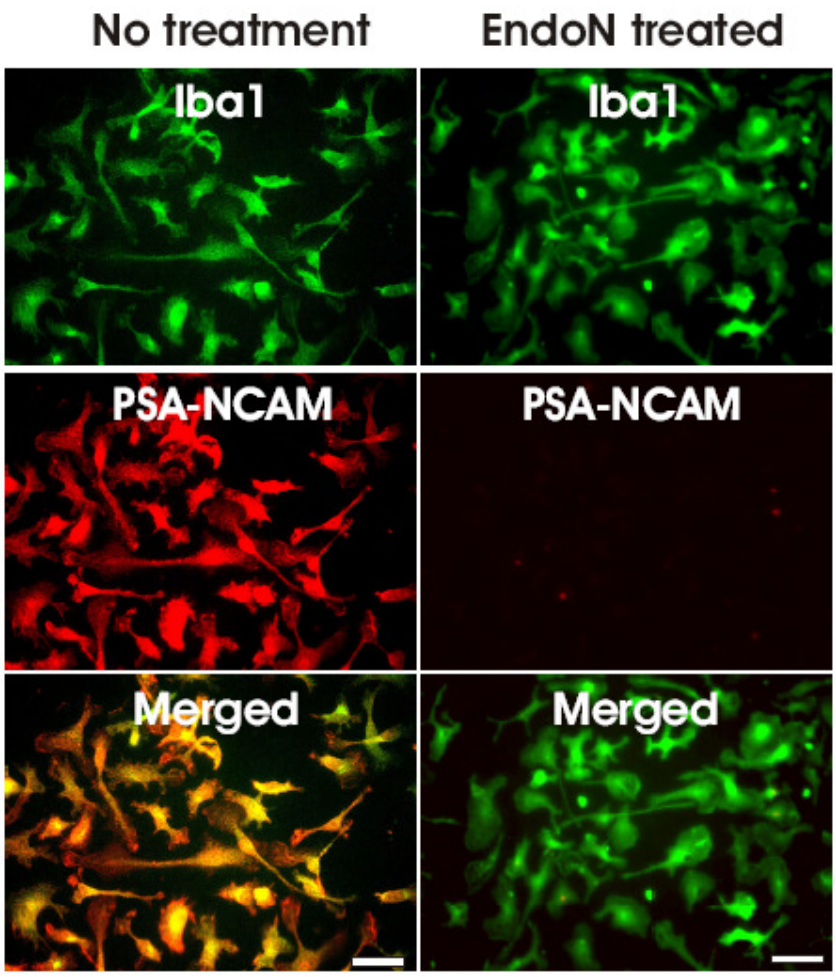


Figure 3-11. Immunostaining of PSA-NCAM on cultured primary microglia. The mouse monoclonal anti-PSA-NCAM antibody and Cy3 conjugated secondary antibody were used to stain the PSA-NCAM expression on primary microglia. Microglia were doubled stained with anti-Iba1 antibody and FITC conjugated secondary antibody. One the right panel, EndoN was applied the culture for 5 hours, which removed the PSA-NCAM from the microglia. Scale bar: 50 μm.

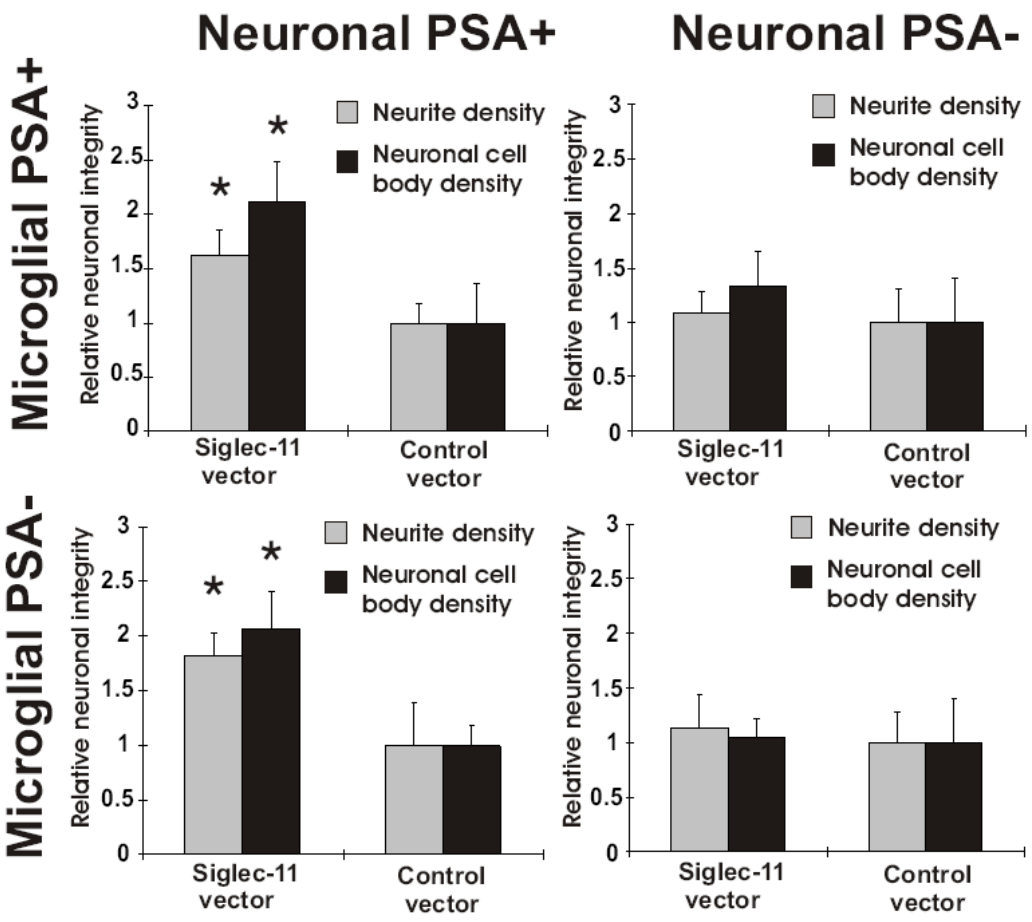


Figure 3-12. Co-culture of primary microglia with neurons. Microglia were lentivirally transduced with Siglec-11 or the control vector. PSA was removed from neurons and/or microglia by EndoN treatment for 5 hours at 37 ^{0}C. Cells were co-cultured for 48 hours. The fluorescence intensity of neurites stained with β-tubulin III was measured and statistically analyzed. Data are shown as mean +/- SEM, N=5, *: P<0.05.

3.5 Generation of Siglec-11 expressing transgenic mice

3.5.1 ES cell-embryo aggregation

3.5.1.1 Establishment of ES cell lines for transgenic mice

The MPI ES cell line and Bruce4 ES cell line were transduced with lentiviral particles carrying Iba1-Siglec-11. 48 hours post-transduction cells were selected in G418 for 10 days. Colonies that survived were picked manually, and insertion of the vector was

verified by PCR amplification of the neomycin marker. Three lines of MPI ES cells (MPI-Siglec-11 ES cells) and 15 lines of Bruce4 ES cells (Bruce4-Siglec-11 ES cells) positively transduced with the transgene were established (Table 3-2).

Table 3-2. Establishment of Siglec-11-ES cell lines

ES cell line	vector	Number of established lines
MPI	Iba1-Siglec-11	3
Bruce4	Iba1-Siglec-11	15

3.5.1.2 Generation of chimeric mice

Aggregation experiment was first carried out with the MPI ES cells. ES cells were aggregated to 8-cell-stage CD1 embryos. The non-modified ES cells were used as a control. From the control experiment, 2 chimeras with about 40-50% chimerism according to the hair color were generated. And one of these 2 mice showed germline transmission. Aggregation of MPI-Siglec-11 ES cells resulted in in total 8 chimeras with chimerism differing from about 20% to 80%, but none of these chimeras gave germline transmission. From the Bruce4-Siglec-11 ES cells, in total 17 chimeras were generated ranging from low chimerism (about 5%) to high chimerism (90%) (Figure 3-13). Unfortunately, also no germline transmisstion were established from these animals (Table 3-3).

Table 3-3 ES cell-embryo aggregation results

ES cell line	Vector	Number of chimeras	Percentage of Chimerism	Germline transmitter
MPI	no	2	40-50%	Yes
MPI-Siglec-11	Iba1-Siglec-11	8	20-80%	No
Bruce4-Siglec-11	Iba1-Siglec-11	17	5-90%	No

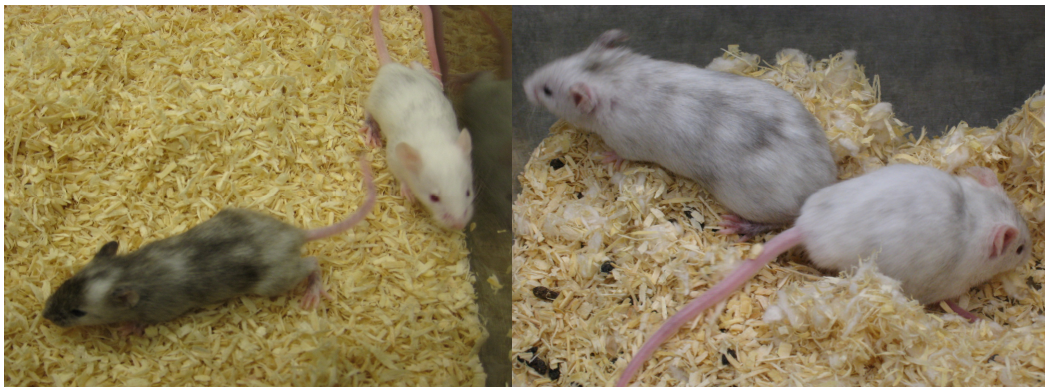


Figure 3-13. Representive photos of Siglec-11 chimeric mice generated from ES cell-embryo aggregation experiment with chimerism ranging from 5% to 90% according to the color of the hair.

3.5.2 Transgenic mouse strains generated by pronuclear injection

PLL-Iba1-Siglec-11 plasmid was digested with restriction enzymes BsiEI and Not1 to separate the Iba1 promoter, the cDNA of Siglec-11 together with the WPRE sequence from the plasmid backbone. The WPRE was kept to replace the function of poly A signal. The purified DNA fragment was injected into B6D2 F1 (F1 generation of DBA and C57/Bl6) mice zygotes. In total 21 founders were established according to genotyping by PCR (Table 3-4).

Table 3-4 Generation of transgenic mice expressing Siglec-11 by pronuclear injection

Zygote background	Gene construct	Founder		Germline transmission
B6D2 F1 (F1 generation of DBA + C57 Bl6)	Iba1-Siglec-11-WPRE	Male	Female	Yes
		11	10	

Ten of the founders were bred to C57/Bl6 mice. Germline transmission of Siglec-11 in the new generation was comfirmed by genotyping. The expression of Siglec-11 mRNA was confirmed by RT-PCR of the brain tissue. Furthermore, Western blot analysis verified that Siglec-11 was expressed in the brain tissue of transgenic mice (Figure 3-14).

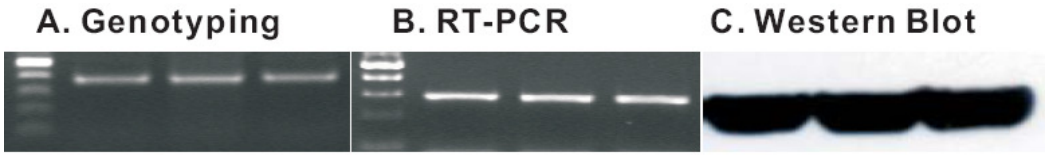


Figure 3-14. Analysis of 3 representative strains of the F1 generation of the Siglec-11 expressing transgenic mice. A. Genotyping of Siglec-11 from the mouse tails. B. RT-PCR analysis of the mRNA expression of Siglec-11 in the brain tissue. C. Western blot analysis of Siglec-11 in the brain tissue.

4 DISCUSSION

4.1 Detection of a splice variant of Siglec-11 in the human brain

In 2002, Siglec-11 was first described by Angata and coworkers (Angata, Kerr et al. 2002). In the search for novel Siglec candidates in human genomic DNA sequences, they identified a Siglec-like putative gene which was proven to be actively transcribed. They isolated the full-length coding region of the cDNA of this gene, which was denoted as Siglec-11, by PCR from a human fetal liver cDNA library and obtained the sequences of untranslated regions by 3'- and 5'- rapid amplification of cDNA ends (RACE). The structure of Siglec-11 was then digged out as having five extracellular Ig-like domains (one V-set Ig-like domain followed by 4 C-set Ig-like domains), a single-pass transmembrane domain, and a cytosolic tail. It contains almost all of the defined features of Siglecs including conserved amino acids (an Arg residue and an aromatic amino acid near the N terminus), and three conserved cysteine residues in the first and second Ig-like domans. By RT-PCR analysis of human multiple tissues cDNA panel using Siglec-11 specific primers, they showed that the expected 400-bp band was prominent in cDNA from brain, placenta, lung, liver and pancreas, but undetectable in heart, skeletal muscle, and kidney. When using a monoclonal antibody 4C4 against Siglec-11 developed by themselves to analyze the protein expression in human tissues, they identified low but distinct expression of Siglec-11 in Kupffer cells in liver, intestinal lamina propria macrophages, brain microglia, and perifollicular cells in spleen, as well as in cells from tonsil and appendix, in a pattern similar but not completely overlapped to that of CD68.

Based on their findings, we set out to study the function of Siglec-11 on brain microglia. We designed Siglec-11 specific primers from different exons from the coding region to verify the transcription of Siglec-11 in human brain tissue. However, the transcript we obtained from human brain tissue was different from what was reported by Angata and coworkers. A 288-bp long exon coding the 5th extracellular Ig-domain (the last C-set Ig-like domain next to the transmembrane domain) was missing. This was consistent in all of the three samples from different patients we analyzed. Interestingly, the cDNA

sequence of Siglec-11 which was derived from human placenta that we obtained from the German genetic material source center was identical to our finding. When we compared the protein size of the human brain Siglec-11 with the Siglec-11 overexpressed in 293 using the short version of cDNA we obtained, there was no difference between them as determined by Western blot analysis. This comfirmed that the splice variant of Siglec-11 (variant 2) we identified was expressed in all the human brain samples we were able to examine. After checking literature, indeed, the shorter version of Siglec-11 cDNA was reported somewhere else previously (Clark, Gurney et al. 2003). The reason why Angata and coworkers did not detect this variant was due to their design of the primers for RT-PCR. Actually, their primers were derived from the sequence coding the intracellular domain and the 3'- untranslated region of Siglec-11 cDNA, while we used primers derived from various exons including not only the extracellular domains but also the intracellular domain, which enabled us to detect the variation in the transcript of Siglec-11.

However, it is also possible that the transcription of Siglec-11 differs in different tissue. The used brain tissue and the obtained placenta cDNA might have a shorter transcript, but other tissues which we did not analyze further might have a longer transcript of Siglec-11. From a structural point of view, the 5th Ig-like domain missing in the detected splice variant 2 of Siglec-11 does not seem to be a key factor affecting the function of Siglec-11. The so far known key elements of the Siglecs are mainly the N-terminal first V-set Ig-like domain, which recognizes and binds sialic acid, the second C-set Ig-like domain which might be required for effective recognizing of sialic acid by the V-set domain, and the intracellular tail which contains the ITIMs that might be involved in the signal transduction. The V-set like Ig-like domains are generally thought to only have the function of pointing the V-set domain away from the cell surface. Thus, it was reasonable for us to use the cDNA of the shorter variant of Siglec-11 in our further study.

4.2 Siglec-11, an inhibitory immune receptor?

Siglecs are emerging as important regulators of the immune system. One prominent

feature of the CD33 related family members including Siglec-11 is the two conserved ITIM-like motifs in the cytoplasmic regions. Numerous studies have showed that the ITIM containing Siglecs could function as inhibitory receptors that modulate leukocyte behaviour, including inhibition of cellular proliferation, induction of apoptosis, inhibition of cellular activation, induction of pro-inflammatory cytokine serection, and suppression of interferon-α production (Crocker, Paulson et al. 2007).

4.2.1 Regulatory function of Siglec-11 on microglia

Microglia have been implicated as active contributors to neuron damage in neurodegenerative diseases, in which the overactivation and dysregulation of microglia might result in disastrous and progressive neurotoxic consequences.

Our study of Siglec-11 was focused on microglia, the brain macrophage. It was reported that in peripheral blood cells Siglec-11 was not detectable (Angata, Kerr et al. 2002) or only at a very low expression level (Nguyen, Hurtado-Ziola et al. 2006) by FACS. We check the expression of Siglec-11 in a human monocytic cell line. Indeed, no Siglec-11 was detected. However, when we differentiate this cell line into macrophage, Siglec-11 transcript was detected by RT-PCR. It is known that under some disease conditions, bone marrow derived monocytes could be recruited to the brain and differentiated into microglia. The induction of Siglec-11 in macrophages differentiated from monocytes implies a specific function of Siglec-11 in microglia/macrophage.

LPS is the endotoxin derived form Gram-negative bacterial and is commonly used to mimic the infection condition. It is reported that LPS induces microglial activation in vivo and in vitro. In our study, under the stimulation of LPS, primary microglia transduced with Siglec-11 showed reduced expression of pro-inflammatory cytokines, namely IL-1β and NOS2, when a flag antibody was used to crosslink the Siglec-11, indicating an inhibitory effect of Siglec-11.

The ability of phagocytosing the corpses of apoptotic cell is one of the key features of microglia. It was reported that glial phagocytic activity is mediated by ITAM signaling transducted by Src and Syk family kinase signaling (Ziegenfuss, Biswas et al. 2008).

There was also evidence that phagocytosis of apoptotic bodies by macrophages was inhibited with sialooligosaccharide ligands of siglec-5 and monoclonal antibodies (mAbs) to siglec-5 (Rapoport, Sapotko et al. 2005). In our study, the Aβ phagocytosis ability was impaired in Siglec-11-expressing primary microglia. Since the cytosolic tail of Siglec-11 was very closely related to that of Siglec-5, which contains two ITIM motifs, it is possible that activation of these Siglecs triggered ITIM-mediated signalling which counteracted the ITAM signaling and lead to the inhibition of the phagocytosis.

Activated microglia are known to be toxic to neurons by releasing a wide range of factors including glutamate, TNF-α, nitric oxide (NO) and IL-1β, which can actively trigger apoptosis in neuronal cell cultures (Bessis, Bechade et al. 2007). Here, we showed that Siglec-11 expressing microglial cells seem to have a less toxic impact to the integrity of neurons. This is consistent to our findings that Siglec-11 could inhibit the secretion of some of these factors such as IL-1β, and NOS2.

4.2.2 ITIM mediated signaling in Siglecs

The importance of balancing positive and negative signals within the immune system is an emerging topic of discussion. When cellular activation is triggered by receptors with ITAMs, counteracting inhibitory signals are delivered through receptors bearing ITIMs. Following phosphorylation by Src-family kinases, ITIMs recruit phosphatases, either Src homology 2 domain-containing inositol polyphosphate 5' phosphatase (SHIP), or more commonly SHP-1 and SHP-2. These phosphatases inhibit signaling pathways by distinct mechanisms, resulting in raised activation thresholds. The presence of two conserved ITIM-like motifs in the cytoplasmic regions of CD33-related Siglecs and the differential expression of these proteins on leukocytes suggests a role in regulating cellular activation. Functional evidence that Siglecs can mediate inhibitory signals has been obtained using mAbs to co-crosslink CD33 or mSiglec-E with an activating human receptor, FcγR1. This resulted in reduced Ca^{2+} influx compared with crosslinking FcγRI alone. Similarly, Siglec-7 was identified as an inhibitory NK-cell receptor in a redirected killing assay in which anti-Siglec-7 mAb was used to cluster Siglec-7 at the interface between NK cell

and target cell. In other functional studies, the addition of intact anti-CD33 or anti-Siglec-7 mAbs to hematopoietic cell cultures led to reduced cell growth and prevented the development of DCs (Crocker and Varki 2001).

Siglec-11 has been shown to be able to recruit SHP-1 and SHP-2. When mouse macrophage-like cells RAW 264.7 stably transfected with Siglec-11 were treated with pervanadate, a potent inhibitor of tyrosine phosphatases, tyrosine phosphorylation was clearly evident, and this was accompanied by co-immunoprecipitation of both SHP-1 and SHP-2. And low levels of SHP-2 could even be seen in immunoprecipitates from non-pervanadate-treated cells, under conditions where phosphorylation of Siglec-11 was undetectable (Angata, Kerr et al. 2002). Our findings that human Siglec-11 expressed in microglia inhibited phagocytosis, reduced pro-inflammatory cytokine transcription and prevented neuronal damage enabled us to assign Siglec-11 as an inhibitory receptor in the immune system, in which ITIM signal transduction might be an important player. However, ingenious experiments need to be carried out to further comfirm the contribution of ITIM signaling.

4.3 PSA, an endogenous ligand of Siglec-11?

Siglec-11 has previously been shown to bind weakly but specifically to α-2–8-linked sialic acids (Neu5Ac-alpha 2-8), but the ligand molecule modified by 2-8-linked sialic acids and recognized by Siglec-11 has not been identified (Angata, 2002). PSA, which is present prominently in the nervous system, emerges as a possible ligand candidate for Siglec-11.

PSA is the long linear homopolymers of α-2,8-linked Nacetylneuraminic acid (Neu5Ac-alpha 2-8). In mammalian cells, most PSA is associated with NCAM (Cremer, Lange et al. 1994). Expression of PSA is abundant in embryonic nervous system and is drastically reduced in the adult. PSA modification of NCAM during neuronal development has been shown to play a significant role in cell migration, axonal guidance, synapse formation, and functional plasticity by preventing the formation of stable cell contacts mediated by NCAM and other cell surface molecules (Bonfanti 2006). Although

overall PSA levels are greatly reduced in the adult brain, high levels of PSA persist in distinct regions that retain neurogenic capacity, such as the SVZ (Rousselot, Lois et al. 1995) and the granule cell layer of the hippocampus (Seki and Arai 1991), or that exhibit physiological plasticity, such as regions of the hypothalamus, the entorhinal–hippocampal complex, the thalamus, the habenular nuclei, the mesencephalic central grey, the lateral geniculate nucleus and dorsal spinal laminae (Seki and Arai 1993; Bonfanti 2006). Perturbation of PSA levels has been shown to influence a wide range of CNS functions and PSA is revealed to be associated with cellular elements that are known to be directly involved in behavioural plasticity. Indeed, altered PSA levels are associated with various neuropathological conditions (Rousselot, Lois et al. 1995), including chronic stress (Pham, Nacher et al. 2003), Alzheimer's disease (Mikkonen, Soininen et al. 1999), schizophrenia (Barbeau, Liang et al. 1995) and temporal lobe epilepsy (Mikkonen, Soininen et al. 1998).

When studying possible ligands of Siglecs, one should always be cautious that these molecules might work both "in cis" and "in trans". Although little PSA has been found in adult non-neural tissues, the immune system is the exception. For example, PSA is reported to be associated with dendritic cells, where expressed neuropilin-2 is polysialylated and influences the activation of T cells (Curreli, Arany et al. 2007). PSA is also found on the surface of natural killer cells (Moebius, Widera et al. 2007). Using a monoclonal antibody specific to PSA-NCAM, we also identified the expression of Siglec-11 in neonatal microglia.

With the help of EndoN, which specifically removes PSA from NCAM, we were able to show that Siglec-11 appeared to have protective effect on neurons, which was associated with the presence of PSA on the neurons, but not on microglia. Thus, in our system, Sigelc-11 and PSA interact in a trans way, but not in cis.

The common sialic acids of mammalian cells are Neu5Ac and Neu5Gc. Humans lack Neu5Gc owing to a mutation in the CMAH (cytidine monophosphate-N-acetylneuraminic acid hydroxylase) gene, which encodes the enzyme required for the conversion of Neu5Ac to Neu5Gc (Sonnenburg, Altheide et al. 2004). The loss of Neu5Gc in human has been proved to may alter biological processes of the siglecs, including siglec-1, and possibly, siglec-4a or -5 (Brinkman-Van der Linden, Sjoberg et al. 2000). Siglec-11 is a

receptor evolutionary appeared very late. In this regard, one can also postulate that Siglec-11 might adapt to the abundant Neu5Ac in the human CNS. Interestingly, PSA does not exist in invertebrate (Rutishauser 2008), which share many fundamental aspects of vertebrate neuronal function and circuitry. Given the correlation of PSA with physical and structural plasticity, it seems likely that the evolution of a less hardwired and more adaptive CNS has taken the advantages of PSA, thereby improving the ability to respond to changing environments.

4.4 Transgenic mice expressing Siglec-11, a tool to study Siglec-11

In the effort to generate transgenic mice that express Siglec-11 specifically in brain microglia and tissue macrophages, we first tried the ES cell-mediated technology. However, the chimeras generated from the aggregation of modified ES cells and mouse embryos did not show gremline transmission ability. And the chimeras themselves were not ideal to study. So we applied the pronuclear injection technology later. Quite a few of founders were successfully generated. And by breeding of the founders to C57/BL6 mice, we have established more than five strains of transgenic mouse lines expressing Siglec-11 in the brain which are valuble tools to study the function of Siglec-11.

4.4.1 A source of Siglec-11 expressing cells for in vitro study

The research on the function of microglial Siglec-11 was aggravated due to the difficulty of getting human microglial cells. Although mouse lacks the ortholog of Siglec-11, mouse microglia still would be a good system to study the signaling via Siglec-11 and the resulting effects since the intracellular signaling pathways are almost identical between mice and humans. Primary microglia expressing Siglec-11 without additional genetic modification steps such as viral transduction would save time and energy from the tedious but also critical preparation of the virus and the subsequent transduction. Furthermore, the difference of the cells used from experiments to experiments will be minimized. Data obtained from the in vitro study on these cells would provide insightful indication to the

features of Siglec-11.

4.4.2 Facilitating the study of Siglec-11 in vivo

As a human specific gene, Siglec-11 is found only in human and its unique expression pattern adds difficulty to the employment of human cells for analyzing its natural functions. However, the Siglec-11-expressing transgenic mice will make the functional study of Siglec-11 in vivo feasible. Particularly, the transgenic mice expressing Siglec-11 in microglia facilitate the study of neuronal immunological diseases such as MS, AD, since mouse models are already available. For example, experimental autoimmune encephalomyelitis (EAE) can be induced in the Siglec-11 transgenic mice to see whether Siglec-11 has any impact on the disease process. Data obtained then will elucidate the functions of Siglec-11 in brain micrglia and its possible impact on the development or therapy of inflammatory and degenerative CNS diseases.

5 SUMMARY

Siglec-11 is a recently identified human-specific CD33-related Siglec expressed on microglia. The full-length cDNA of Siglec-11 encodes 5 extracellular Ig-like domains, a single pass transmembrane domain, and a cytosolic tail, which contains ITIMs. In human, histopathology demonstrated the expression of Siglec-11 on tissue macrophages in various tissues, including microglia in brain. We studied Siglec-11 in microglia. A Siglec-11 splice variant, but no full length Siglec-11 was identified in human brain tissue samples. Functional analysis was performed in cultured mouse microglial cells lentivirally transduced with this splice variant of human Siglec-11. Under stimulation with LPS, gene transcription of IL-1β and NOS2 of microglia was reduced after cross-linking of Siglec-11. The Aβ phagocytosis ability was impaired in Siglec-11 expressing microglia. PSA-NCAM as a putative ligand of Siglec-11 was detected on microglia and neurons. Co-culture of microglia expressing Siglec-11 and neurons demonstrated neuroprotective function of Siglec-11. Neurite density and neuronal cell body density were higher in co-cultures with Siglec-11 expressing microglia than those of control co-cultures. Neuroprotective effect was dependent on sialic acid residues on neurons, but independent on polysialylated residues of microglia. Transgenic mice were generated expressing Siglce-11 under the microglial Iba1-promoter. Chimeric mice were obtained from aggregation of genetically modified ES cells and embryos, but no germline transmission was achieved. Germline transmission was obtained from pronuclear injection of Siglec-11 DNA. Several strains of transgenic mice expressing Siglec-11 in the brain have been sucessfully established. Thus, data show that Siglec-11 is an inhibitory receptor of microglia that might help to create an immunosuppressive milieu in the CNS and alleviate microglial neurotoxicity. Humanized transgenic mice expressing Siglec-11 we have generated serve as a good model to provide valuble information on the natural features of Siglec-11.

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8 ERKLÄRUNG/DECLARATION

Hiermit versichere ich, dass diese Dissertation von mir persönlich, selbständig und ohne jede unerlaubte Hilfe angefertigt wurde. Die vorliegende Arbeit wurde an keiner anderen Hochschule als Dissertation eingereicht. Ich habe früher noch keinen Promotionsversuch unternommen.

This thesis has been written independently and with no other sources and aids than stated.

Bonn, March 2009

Yiner Wang

9 CURRICULUM VITAE

Publications

Katrin Kierdorf, **Yiner Wang,** and Harald Neumann. Immune-Mediated CNS Damage. Results Probl Cell Differ. 2009 Jan 8. [Epub ahead of print].

Yiner Wang, Xi Chen, Weiquan Zhu, Hao Zhang, Shengshou Hu and Xiangfeng Cong. Growth Inhibition of Mesenchymal Stem Cells by Aspirin: Involving of the Wnt/beta-catenin Signal Pathway, *Clinical and Experimental Pharmacology and Physiology* (2006) **33**, 696–701

Zhao Yanhong, Chen Xi, Cong Xiangfeng, Wang Shuyu, **Wang Yiner**, Liu Lisheng. Effect of the Pro12Ala polymorphism of peroxisome proliferator activated receptor γ2 gene on obesity in North China, Chinese Journal of Laboratory Medicine, 2006 Vol.29 No.10 P.918-922

Wang Yiner, Hu Shengshou, Chen Xi, Cong Xiangfeng. Influence of aspirin on the growth of mesenchymal stem cells and the possible pathway of its role, Chinese Journal of Clinical Rehabilitation, 2005 Vol.9 No.19 P.74-76

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Yiner Wang and Harald Neumann. Alleviation of neurotoxicity by microglial human Siglec-11.

Sadanand Gaikwad, Sergey Larionov, **Yiner Wang**, Holger Dannenberg, Takashi Matozaki, Alon Monsonego, Dietmar R. Thaland Harald Neumann. Signal regulatory protein- 1: a microglial modulator of phagocytosis in Alzheimer's disease

Published abstracts and congress contributions

Yiner Wang and Harald Neumann, Silencing function of human Siglec-11 in microglia. DFG-Klinische Forschergrppe 177 Kick off meeting on "Innate immunity in chronic neurodegeneration", Bonn 2009.

Yiner Wang and Harald Neumann, Silencing function of human Siglec-11 in microglia. 13th Semester Meeting des Bonner Forum Biomedizin, Bad Breisig, 2009.

Yiner Wang and Harald Neumann. Human-specific, microglial receptor Siglec-11 in neuroinflammatory diseases. 11th Semester Meeting des Bonner Forum Biomedizin, Bad Breisig, 2007.

Additional Information

Languages: Chinese (mother tongue), English (fluent), German (basic)

Computer programs: Word, Excel, Power Point, EndNote, Adobe Photoshop, Image J, CorelDRAW, Cell Quest Pro, FlowJo, NTI vector, Primer Premier 5.0 etc.