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The Fascinating World of SigLec Proteins: A Primer

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INTRODUCTION

Sialic acid-binding immunoglobulin-like lectins (Siglecs) are a family of transmembrane proteins able to recognize sialic acids, nine-carbon atoms sugars highly expressed on the cell surface and one of the major components of the glycocalix. Sialic acids usually occur at the terminal positions of oligosaccharides and glycoconjugates, and they can act as ligands for receptor recognition (trans-interactions) or masking recognition sites on the same cell surface (cis-interactions). Sialic acids occur under differently substituted forms, all derived from the neuraminic acid (Neu) parent molecule, like for example, the N-acetylated variant (Neu5Ac), which is the most abundant, or the N-glycolylated derivative (Neu5Gc), which cannot be synthesized by humans (Figure 1).

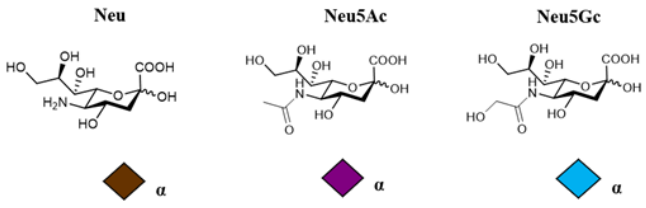


Figure 1. *The most abundant sialic acids : the neuraminic acid and its N-acetyl and N-glycolyl derivatives (Neu5Ac and Neu5Gc, respectively). They are shown in their α-configuration, as if they were in their bound states. The colored squares represent their standard graphic symbols. (Varki et al., 2016)*

Free sialic acids are in the energetically preferred β-form, but they adopt the α-anomeric configuration when bound.(Schauer & Kamerling, 2018 ; Varki, 2001) Because of their position on the surface of the membranes, sialic acids are mainly available for protein-ligand interactions, mediating several physiological and pathological processes.(Engin et al., 2020 ; Gianchecchi et al., 2021 ; Heida et al., 2021 ; Aubli et al., 2020 ; Varki, 2008 ; Wang, 2012) As mentioned above, Siglecs are one family of proteins that mediate these interactions.

Depending on their expression site, they are involved in many different processes. For this reason, in the last years, Siglecs have emerged as potential therapeutic targets for treating several diseases: the main areas include autoimmunity, infections, inflammation, ageing, and cancer.

The main goal of this chapter is to review all the most recent discoveries about Siglecs. In every section, the main characteristics of each Siglec will be summarized, from their biological and pathological role to the structural information available. Besides, the main essential strategies exploited to target them will also be explained, from the traditional approach with small molecules to the development of multivalent compounds and antibodies.

THE SIGLECS FAMILY

The numbering of Human Siglecs follows the order of their Tdiscovery. The representatives murine Siglecs that are not homologous to the human ones are designed by a letter (e.g. Siglec-F). (Varki et al., 2015) Human Siglecs are divided into two groups depending on their homology and evolutionary conservation (Figure 2). CD22-related Siglecs share only 25-30% of sequence identity, and they have true orthologs in other mammalian species : Sialoadhesin (Siglec-1), CD22 (Siglec-2), myelin-associated glycoprotein (MAG, Siglec-4), and Siglec-15 belong to this group. The second group consists of CD33-related Siglecs (CD33rSiglecs). Among these, Siglec-12 lost its ability to recognize sialic acid, and it was renamed Siglec-XII,

while the Siglec-13 gene is not present in humans ; therefore, they will not be discussed in the following sessions. (von Gunten & Bochner, 2008) CD33rSiglecs are characterized by high sequence homology and minor conservation between species.(Angata et al., 2004 ; Bornhöfft et al., 2018 ; Crocker et al., 2007) CD33rSiglecs are also expressed in mice, but they represent paralogs and not true orthologs of the human counterparts : Siglec-F and Siglec-G correspond to Siglec-8 and Siglec-10, respectively. (Jandus et al., 2011) The high evolutionary differentiation might respond to different mechanisms of evasion from the immune system used by pathogens or cancer cells, like mimicking our self-glycans.(Angata, 2006 ; Macauley & Paulson, 2014 ; Varki & Angata, 2006)

Structurally, Siglecs present a V-set domain on their extracellular N-terminal part, containing the sialic acid-binding pocket, and a variable number of C-2 set domains (from 1 to 16), both immunoglobulin (Ig)-like domains. In their resting state, Siglecs are generally engaged in cis-interactions with sialic acid ligands expressed on the same cell surface. Sialoadhesin is an exception : due to its length (16 C-2 set domains) it is mainly involved in trans-interactions. (Crocker et al., 2007 ; Hartnell et al., 2001 ; Nakamura et al., 2002) High-affinity ligands can compete with the cis-ligands and bind to Siglecs, as shown with CD22. (Collins et al., 2006)

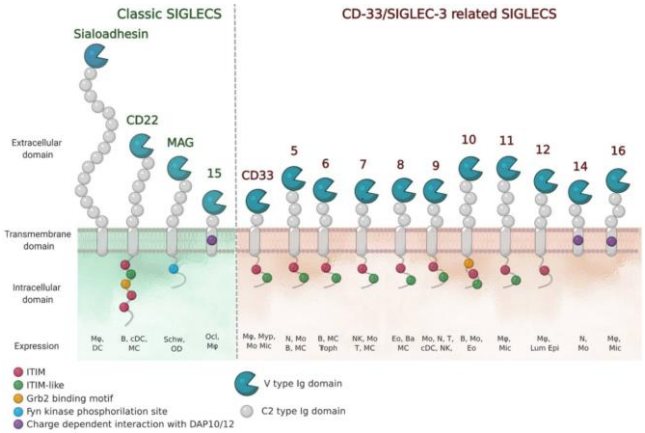


***Figure 2**. Structural features of human Siglecs and their expression. Mø, macrophages ; DC, dendritic cell ; B, B cells ; MC, mast cells ; Schw, Schwann cells ; OD, oligodendrocytes ; Ocl, osteoclasts ; Myp, myeloid progenitor ; Mo, monocytes ; Mic, microglia ; N, neutrophils ; Troph, trophoblasts ; NK, natural-killer cells ; T, T cells ; Eo, eosinophils ; Ba, basophils ; Lum epi, lumen epithelia cells). Figure from Lenza et al.(Lenza et al., 2020)*

The signaling is initiated by the intracellular regulatory motifs, except for Sialoadhesin that does not contain any domain in this region : most of the Siglecs present an immunoreceptor tyrosine-based inhibitory motif (ITIM). Src kinases first phosphorylate these domains, then, Src-homology 2 domain (SH2)-containing phosphatases SHP-1 and SHP-2, once recruited, de-phosphorylate important signaling molecules and initiate the inhibitory cascade. (Pillai et al., 2012) Only Siglecs-14, -15 and -16 have an activating activity. They contain positively charged amino acid residues that bind to the coreceptor DAP12, which in turn contains an immunoreceptor tyrosine activation motif (ITAM) in its cytoplasmic domain. Once the binding to Siglecs takes place, DAP12 initiates the activating signaling. (Cao et al., 2008a ; Takamiya et al., 2013) Generally, activating Siglecs are coupled with inhibitory counterparts : Siglec-5 vs Siglec-14 and Siglec-11 vs Siglec-16, usually expressed on the same cells. (Macauley et al., 2014)

As previously mentioned, all Siglecs bind sugars containing sialic acid, but each with its specificities. They have evolved to recognize all the different ways in which the sialic acids are displayed on glycoproteins and glycolipids to be specific for the type of linkages (α2-3, α2-6, and/or α2-8), or the type of sugars to which the sialic acid is bound to. Usually, Siglecs bind to their natural sialoside ligands with low affinities (0.1-1 mM). (Blixt et al., 2003) The N-terminal V-set domain mediates the binding. It is composed of antiparallel β-sheets with a shallow binding pocket for the sugar and an additional loop in the proximity, which provides more specificity for carbohydrates. One of the most conserved amino acids in this region is an arginine residue which interacts with a salt bridge with the carboxylate of the sialic acid. (Movsisyan & Macauley, 2020)

Siglecs occur mostly on immune cells, except for MAG, which is present on myelinating cells of the nervous system. Depending on the roles and sites of expression, Siglecs are involved in many physiological and pathological processes, especially as immunomodulators. (Büll et al., 2016)

SIALOADHESIN

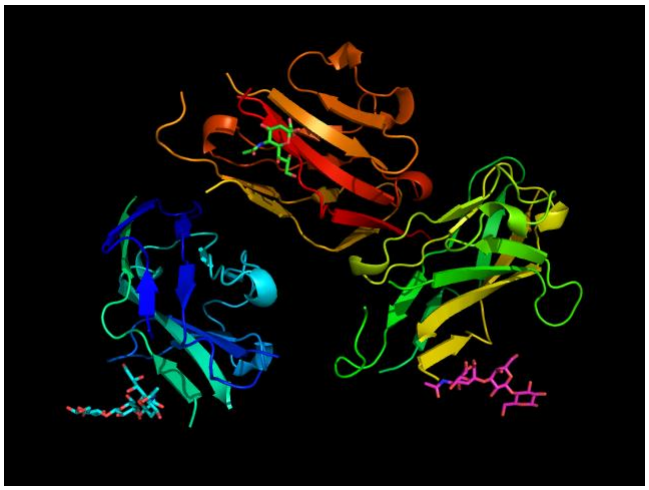
Sialoadhesin (also known as Sn, Siglec-1 or CD169) was one of the first proteins of the Siglec family to be characterized.(Crocker et al., 1994 ; Kelm et al., 1994) It shows a high level of conservation between mouse and human, especially in the extracellular region. It is the only Siglec that presents 17 Ig-like domains outside the cell, including one V-set domain and 16 C-2 set domains. (P.R. Crocker et al., 1991 ; Hartnell et al., 2001) Unlike the majority of Siglecs, it does not have an intracellular domain. Due to the length of the extracellular portion, Siaoloadhesin is more available for trans-interactions with external ligands than cis-interactions with cell-surface molecules. For this reason, it has a principal role in cell-cell interactions and signaling. (Munday et al., 1999 ; O'Neill et al., 2013)

Sialoadhesin occurs mainly on macrophages (described as CD169+ macrophages), playing a crucial role in host protection. Upon recognizing sialic acid epitopes expressed on the cell surface of bacteria or viruses, Sialoadhesin promotes the phagocytic activity of macrophages, optimization of antigen presentation, and following activation of the adaptive immune response. (Chang et al., 2014 ; Chang & Nizet, 2020 ; Klaas et al., 2012)

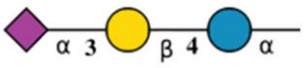
It presents the same role on other antigen-presenting cells (APC) like dendritic cells (DC), where it triggers antiviral immunity taking part in pathogen uptake via endocytosis. Unfortunately, enveloped viruses such as Ebola or human immunodeficiency virus (HIV)-1 have exploited this mechanism to enhance the auto dissemination in tissues and escape the immune system.(Gummuluru et al., 2014 ; Perezzsolt et al., 2019 ; Perez-Zsolt et al., 2019) HIV's capture by DC facilitates virus dissemination and release in cell contact sites with the CD4+ T cell. Therefore, the virus can easily reach these sites, mainly present in lymphatic tissues and trans-infect T-cells. (McDonald et al., 2003 ; Sewald et al., 2015)

Sialoadhesin has also been identified as a potential biomarker for autoimmune diseases : for example, its expression increases in

some pathologies correlated with interferon signaling as systemic lupus erythematosus or systemic sclerosis.(Biesen et al., 2008 ; Eakin et al., 2016 ; York et al., 2007) In particular, Sialoadhesin is associated with an increased expression on peripheral blood monocytes in rheumatoid arthritis. (Xiong et al., 2014)



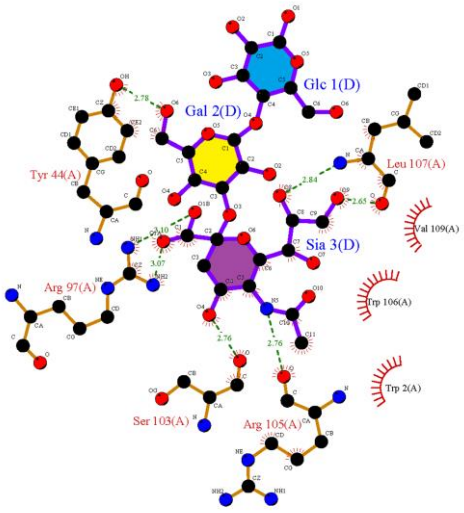
N-terminal domain of sialoadhesin (mouse) in complex with 3'sialyllactos
1qfo



Mass: 633.552

DNeup5Aca2-3DGalpb1-4DGlcpa1-ROH

3'-sialyl-alpha-lactose



WURCS=2.0/3,3,2/[a2122h-1a_1-5][a2112h-1b_1-5][Aad21122h-2a_2-6_5*NCC/3=O]/1-2-3/a4-b1_b3-c2

Figure 3. *(Top) Crystal structure of the N-terminus of Sialoadhesin together with 3'-sialyllactose. PDB code : 1QFO. (Bottom) Main interactions between Sialoadhesin and 3'-sialyllactose. The nomenclature, standard graphical representation and the computational characterization of the oligosaccharide are also presented.*

Sialoadhesin has a preference for α2-3 glycosidic linkage. (Crocker et al., 1999) The crystal structure of the N-terminus of Sialoadhesin together with the 3'-sialyllactose was firstly reported in 1998. The sialic acid almost entirely mediates the binding, and a salt bridge between its carboxylate and Arg97 is essential for the binding. (May et al., 1998) Other essential interactions include one hydrogen bond between OH-4 and the main chain carbonyl of Ser103 and a second one between the amide nitrogen and the main chain carbonyl of Arg105. Hydroxyl groups in positions 8 and 9 form hydrogen bonds with the backbone amine and carbonyl groups of Leu107, respectively. Hydrophobic interactions occur mainly with Trp106 (Figure 3). (May et al., 1998)

The development of small molecules targeting Sialoadhesin started from the methyl-α-Neu5Ac, which displays an affinity in the 1-3 mM range. The subsequent introduction of biphenyl substituents in position 9 of the sialic acid improved the affinity of 13-fold thanks to hydrophobic interactions in a pocket close to the sialic acid-binding site (biphenylcarbamoyl(BPC)-Neu5Ac 2, Figure 4). (Zaccai et al., 2003)

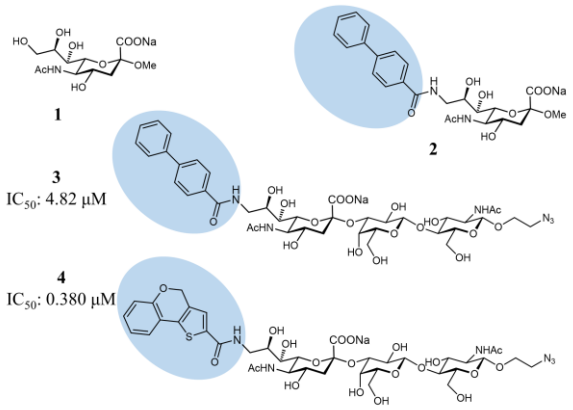


Figure 4. *Development of high affinity and selective ligands for Sialo adhesin. Subsitutents introduced in position 9 of the sialic acid are highlighted in blue.*

Subsequently, liposomes decorated with a related ligand, the 9-N-BPC-Neu5Acα2-3Galβ1-4GlcNAc (3, Figure 4), could target Sialoadhesin-expressing cells, but the interaction was not selective enough for *in vivo* targeting. (Blixt et al., 2003) As a result of these promising results, a virtual screening focusing on position 9 was performed to improve the affinity and selectivity in this region : the best compound identified, the 9-N-(4H-thieno[3,2-c]chromene-2-carbamoyl)-Neu5Acα2-3Galβ1-4GlcNAc 4 (TCC-Neu5Ac, 4, Figure 4) bound selectively to Sialoadhesin with an IC50 of 0.38 µM, measured by competitive binding assay. The same compound, displayed on liposomes, targeted efficiently *in vivo* CD169+ macrophages.(Edgar et al., 2019 ; Nycholat et al., 2012) These liposomes can represent an optimal tool to deliver antigens or drugs to macrophages selectively.

CD22 (SIGLEC-2)

CD22 (Siglec-2) is one of the best described Siglecs. It is well conserved across mammals, and it is highly expressed on B cells, where it regulates their function.

There are seven Ig-like domains in the extracellular region, where the V-set domain contains the sialic acid-binding site, as in every Siglecs. The cytoplasmatic tail contains five domains, three ITIM, one ITIM-like, and one Grb2 binding motif, involved in the negative signaling. (Crocker et al., 2007) CD22 is indeed an inhibitory co-receptor of the B cell receptor (BCR), and it helps set a threshold for the activation of the cell itself. (Tsubata, 2012) To suppress the BCR signals, CD22 should be in its proximity : this will result in phosphorylation of the ITIMs by the SRC-family kinase LYN, the recruitment of SHP1, the dephosphorylation of BCR complex and the following downregulation of the signal (Figure 5).(Nitschke, 2005 ; Tedder et al., 2005) In the resting state, CD22, sialylated, is usually “masked” by cis-interactions with ligands expressed on adjacent CD22 molecules, forming clusters and limiting its association with the BCR. Despite this, CD22 masking does not entirely prevent trans-interactions and the distribution to cell-cell contacts sites where the BCR is also involved.(Collins et al., 2004 ; Enterina et al., 2019 ; Poe et al., 2004)

Considering its inhibitory activity, CD22 is a potential target for treating B cells-associated malignancies or autoimmune disorders, such as hairy cell leukemia, chronic lymphocytic leukemia, and non-Hodgkin lymphoma.(Clark & Giltiay, 2018 ; Dörner et al., 2012 ; Sullivan-Chang et al., 2013a)

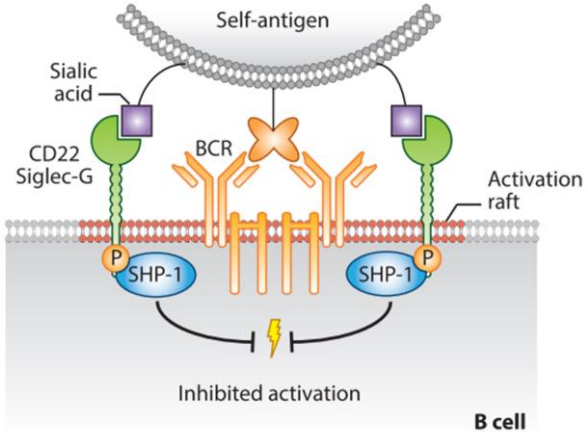


Figure 5. *Colocalization of CD22 with BCR and suppression of BCR signaling. Figure taken from S. Duan et al.(Duan & Paulson, 2020)*

One of the strategies exploited to target CD22 is the use of antibodies. (Angata et al., 2015) The first fully humanized anti-CD22 IgG1antibody is Epratuzamab (Emab) : it does not kill malignant B cells as a single agent, but it showed positive results when associated with Rituximab. (Leonard & Goldenbergm, 2007 ; Sullivan-Chang et al., 2013b) Clinical trials have also been conducted on the use of Emab for the treatment of autoimmune diseases like systemic lupus erythematosus (SLE).(Geh & Gordon, 2018 ; Gottenberg et al., 2018) Since upon antibody (Ab) binding CD22 is internalized, another approach is using Ab-conjugated drugs, such as inotuzumab ozogamicin, where the Ab is conjugated with a drug able to disrupt the DNA. This agent is approved for treating acute lymphoblastic leukemia (ALL). (Kantarjian et al., 2016) Recently, CD22 has been a target for CAR T cell therapies to redirect T cells effectors towards malignant B cells : positive results were obtained for the treatment of B cell ALL. (Adeel et al., 2021)

Different strategies were also adopted for the synthesis of high-affinity ligands for CD22. CD22 has a strong preference for α2-6 Sia ligands, in particular Neu5Acα2-6Galβ1-4GlcNAc (Neu5Acα2-6LacNAc), with an affinity range between 50-200 µM, and α2-6-sialylated 6-sulfo-LacNac.(Kimura et al., 2007 ; Macauley et al., 2015) The 3D structure of CD22 Ig domains 1-3 has been determined (Figure 6). (Ereño-Orbea et al., 2017) The conservative Arg120 is essential ; mutations in this position abrogate the binding. (Van Der Merwe et al., 1996) Other important interactions involve the N-acetamido substituent and the glycerol chain of the neuraminic acid, H-bonded with Met129. A hydrophobic pocket close to position 9 of the sialic acid accommodates large and aromatic substituents. α2-3 linked ligands would clash with the Tyr64, so CD22 is specific for α2-6 linkages.(Kelm et al., 1998 ; Movsisyan & Macauley, 2020)

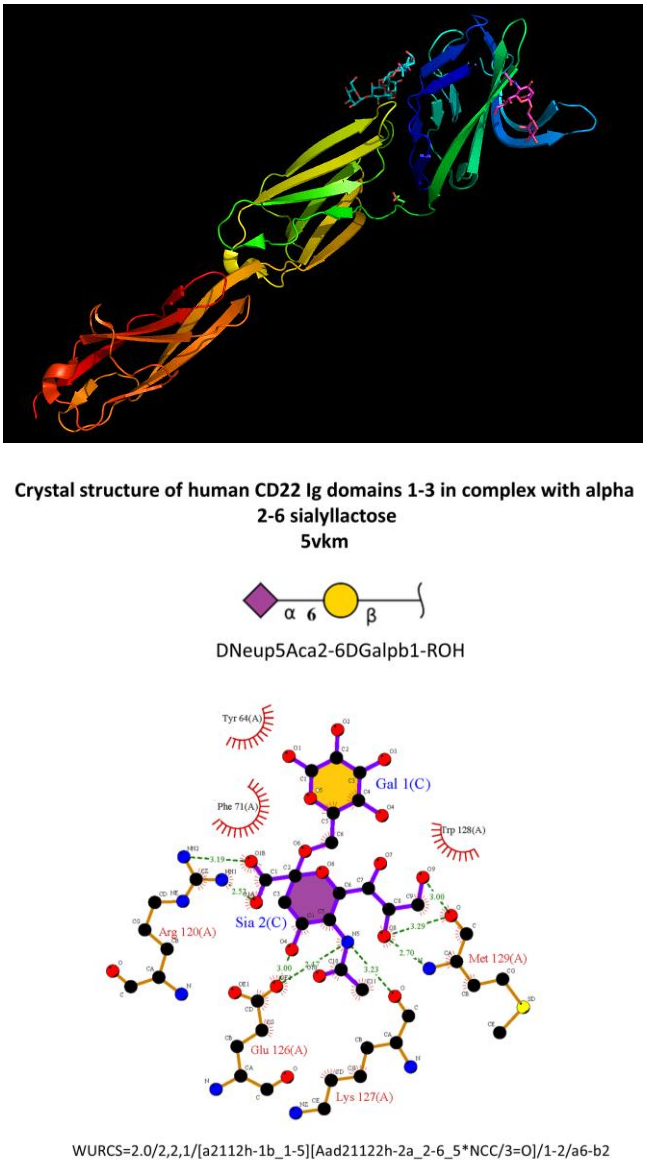


Figure 6*. (Top) Crystal structure of CD22 Ig domains 1-3 in complex with alpha 2-6 sialyl lactose . PDB code : 5VKM. (Bottom) Main interactions between CD22 and α 2-6 sialyl lactose. The nomenclature, standard graphical representation and the computational characterization of the disaccharide are also presented..*

Considering the main interactions, methyl-α-Neu5Ac has been the starting point for initial modifications. As for Sialoadhesin, introducing a biphenyl moiety in position 9 of the sialic acid improved the affinity for CD22. (Kelm et al., 2002) Then, combinations of favorable fragments in different positions of the sialic acid, like positions 2, 4, 5 and 9, led to the discovery of several ligands with high potency and selectivity for CD22 (Figure 7).(Abdu-allah et al., 2011 ; Abdu-Allah et al., 2020 ; Kelm et al., 2013 ; Prescher et al., 2014) Compound 5 was the first monomeric ligand to show high affinity to a Siglec protein, to regulate B cell activation *in vitro* and the immune response *in vivo.*(Abdu-allah et al., 2011 ; Matsubara et al., 2018 ; Sheikh et al., 2018)

High-affinity monovalent ligands can also target CD22 by multivalent presentations. For example, the binding of CD22 with liposomal nanoparticles covered by both antigens and CD22 ligands causes the apoptosis of the B cell in mice and humans : these Siglec-engaging tolerance-inducing antigenic liposomes (STALs) can be exploited to tolerize B cells to specific antigens and to prevent an unwanted immune response.(Macauley et al., 2013)

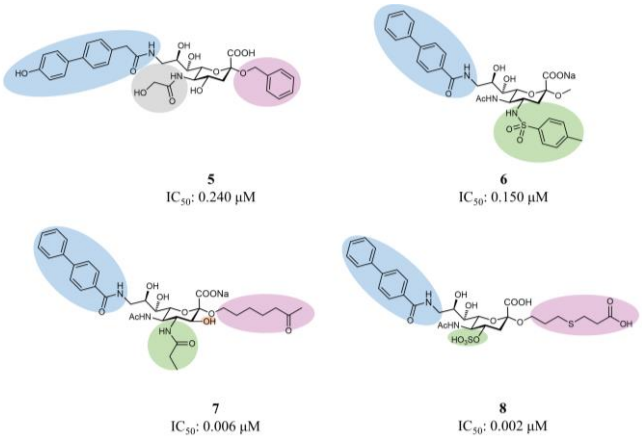


Figure 7*. High-affinity ligands for CD22. Substituents introduced in positions 2, 3, 4, 5 and 9 of the sialic acid are highlighted in pink, orange, green, grey and blue, respectively.*

The endocytic properties of CD22 are used for delivering cargo to B cells. Indeed, liposomal particles can be internalized, CD22 will be recycled to the membrane surface, and the cargo will stay in the cell. (O'Reilly et al., 2011) This strategy successfully delivered doxorubicin to the B cells, resulting in killing B cells lymphoma both *in vitro* and in mice. Besides, a soluble dimeric high-affinity CD22 ligand conjugated to a toxin was efficient in killing B cells ALL.(Chen et al., 2010 ; Schweizer et al., 2012). As an alternative to antibodies or nanoparticles, multivalent ligands conjugated to toxins were efficiently internalized and caused the death of malignant B cells. (Peng & Paulson, 2017)

CD33 (SIGLEC-3)

CD33 (Siglec-3) is one of the first Siglecs discovered, and it is the shortest member of the Siglecs family. The extracellular portion presents a V-set domain, which mediates the interaction with the sialic acid, and one C-2 Ig domain : because of its short length, it is more prone to bind ligands on the same cell surface. (Freeman et al., 1995) Alternative splicing of CD33 RNA leads to a shorter isoform that lacks the V-set domain. (Pérez-Oliva et al., 2011) It exhibits ITIM and ITIM-like domains on the cytosolic tail ; the first one is absent in mouse. As mentioned in the previous sections, these domains are involved in inhibitory signaling with the typical cascade common to other Siglecs. (Paul et al., 2000) Next to SH2-phosphatase, ITIM can also be bound by the suppressor of cytokine signaling 3 (SOCS3). It leads to ubiquitination and consequent degradation of CD33: this mechanism causes the loss of this receptor during an inflammatory process. (Orr et al., 2007) CD33 internalizes upon binding or dimerization, particularly with antibodies : this process decreases the overall general availability of CD33 on the cell surface. (Walter et al., 2008)

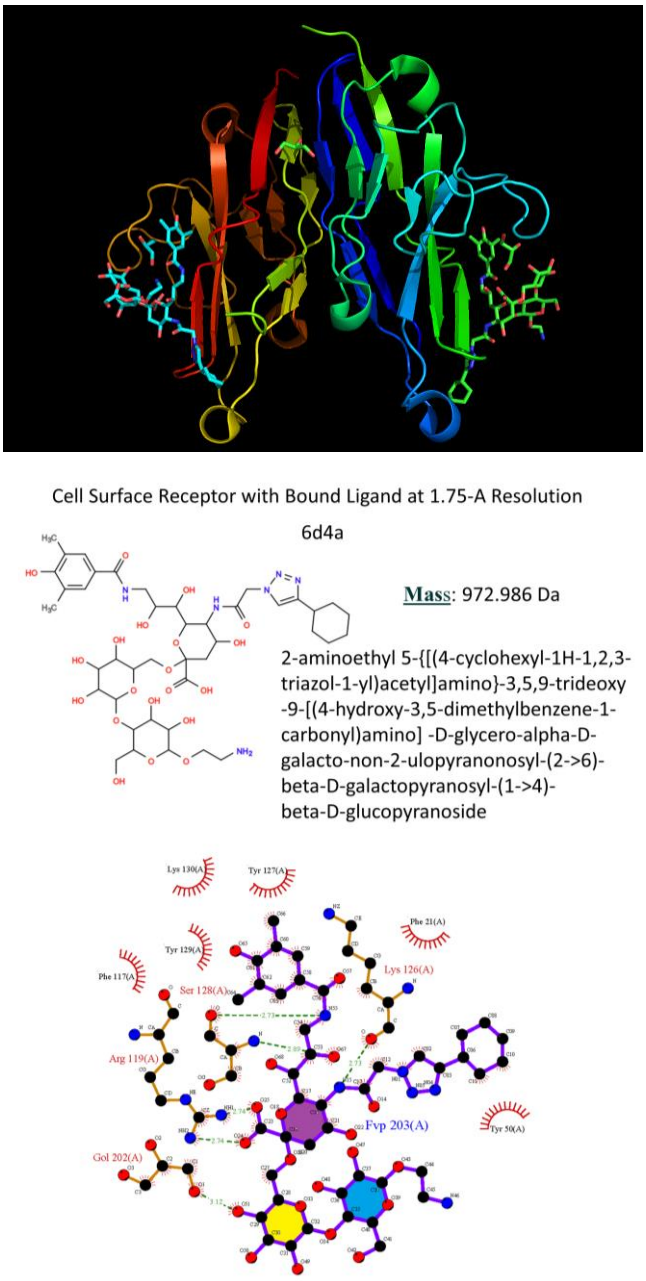


Figure 8. *(Top) Crystal structure of CD33 together with ligand 9. PDB code : 6D4A. (Bottom) Main interactions between CD33 and ligand 9.*

CD33 is expressed on myeloid progenitors, macrophages, monocytes, microglia and granulocytes, regulating phagocytosis and inflammatory responses. (Bhattacherjee et al., 2019 ; Crocker et al.,

2012 ; Macauley et al., 2014) In particular, CD33 has been identified as a marker and target for acute myeloid leukemia (AML) since it is highly expressed on AML blasts. (Ehninger et al., 2014 ; Robertson et al., 1992) Gentuzumab ozogamicin is a CD33antibody-drug conjugate approved by the FDA for the treatment of AML : upon internalization, it releases calicheamicin, a drug able to bind the DNA and cause cell death. (Maakaron et al., 2021)

Dysregulation of microglial phagocytic activity is also connected to Alzheimer's disease (AD), with reduced ability to remove amyloid-beta (Aβ) plaques in the brain. Pieces of evidence show that polymorphisms in the CD33 gene may represent a risk for AD, which would also affect microglial phagocytic activity. However, these studies have been conducted on mice, which presents a different form of CD33. Therefore, these conclusions remain to be confirmed. A recently presented human CD33 knock-in mouse model might answer these open questions. (Griciuc et al., 2013) Besides, an anti-CD33 antibody is in phase I clinical trials to treat AD.

CD33 has a modest preference for α2-6 linkages. The crystal structure of CD33 has been obtained both in apo form and together with ligand 9, identified before the structural information was available (Figure 8). (Miles et al., 2019 ; Rillahan et al., 2014)

As for the other Siglecs, the conservative Arg119 forms a salt bridge with the sialic acid. Around it, two clefts accommodate the hydrophobic substituents in positions 5 and 9. (Movsisyan & Macauley, 2020) Presentation of ligand 9 on microparticles to microglial cells increased the uptake of the toxic peptide amyloid-β involved in the AD. (Miles et al., 2019) Besides, the interaction between liposomes presenting allergen and ligand 9 and mast cells suppressed IgE-mediated mast cells' activation. (Duan et al., 2019) These two recent findings confirm how CD33 represents a very interesting pharmacological target for AD treatment and preventing allergic reactions.

MAG (SIGLEC-4)

MAG (Siglec-4) is the most conserved among Siglecs and, unlike the rest, does not have any involvement in the immune system. Indeed, it occurs on oligodendrocytes and Schwann cells in the central and peripheral nervous systems. (Lopez, 2014). It is a highly glycosylated protein, presenting one V-set domain and four C2-set domains in the extracellular region and, as Sialoadhesin, none ITIM or ITAM intracellular motif. (Quarles, 2007) The cytoplasmatic tail can be of two different lengths leading to two isoforms, the L-MAG and S-MAG. The L-MAG presents a Fyn-kinase phosphorylation site.(Lai et al., 1987 ; Yamauchi et al., 2012) By interacting (trans-interactions) with neuronal gangliosides such as GT1b and GD1a, MAG assures the correct spacing between the axon and the myelin. Controversial results have been obtained regarding MAG's biological function, but different studies support the theory that it inhibits axonal regeneration. Upon myelin disruption, MAG can also diffuse as a soluble form to other sites and significantly inhibit axon growth. This explains why neurons degenerate even in the absence of myelin disruption in the early phases of multiple sclerosis. On the other hand, MAG intervenes in the regulation of myelination, mainly via the activation of the Fyn-kinase. The mechanism behind its functions is not entirely elucidated yet. A study showed how the trans-interaction could explain the bi-directional signaling with the ganglioside and the cis homodimerization observed through the domains Ig4 and Ig5. (Pronker et al., 2016) Like other Siglecs, MAG is an endocytic receptor: via a clathrin-dependent mechanism, it is recycled in the membrane contributing to its modeling. (Winterstein et al., 2008)

Different strategies have been pursued to understand its function and roles better. An anti-MAG humanized monoclonal antibody (mAb) from Glaxo (GSK24932) underwent clinical trials for promoting neuronal regenerations in strokes, blocking MAG inhibitory activity. (Barbay et al., 2015 ; McKerracher & Rosen, 2015) Unfortunately, the positive results observed in squirrel monkeys did not translate in humans : however, there was no direct evidence that the therapy reached the target, so further studies are necessary. (Cramer et al., 2017) Different efforts focused on developing small molecules. (Schwardt et al., 2015) The natural ligands of MAG are α2-3 sialosides which bind to the target protein in the low micromolar range. They are a good starting point compared to other Siglecs, which bind their natural ligands with less affinity.

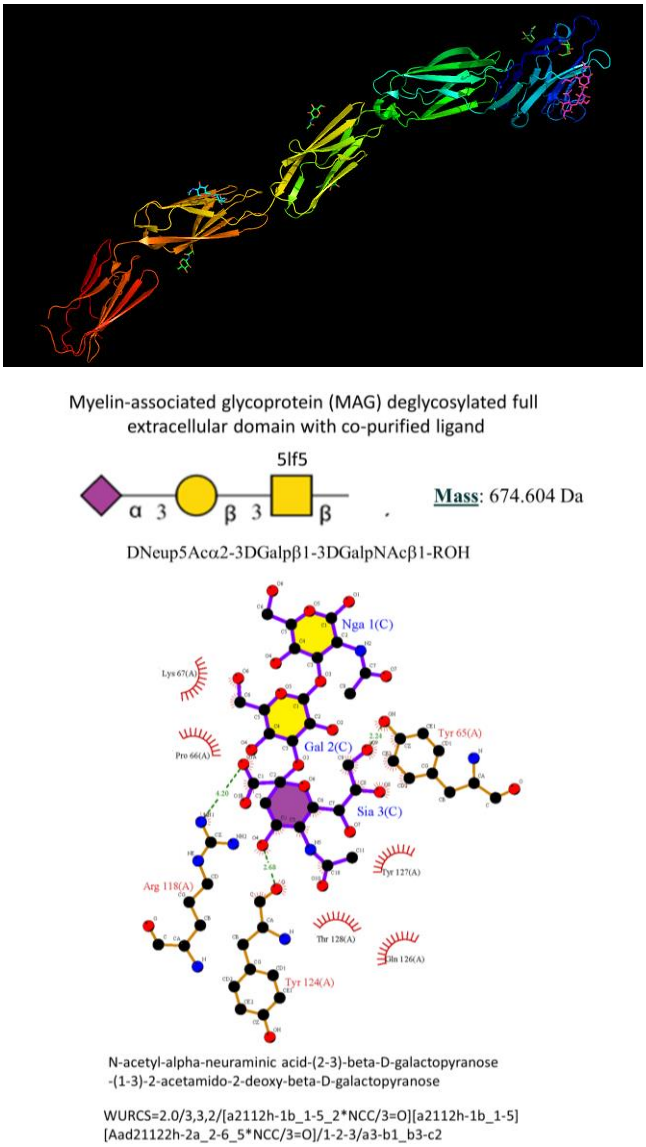


Figure 9. *(Top) Crystal structure of MAG full extracellular domain with a co-purified ligand. PDB code : 5LF5. (Bottom) Main interactions between MAG and 3'-N-Acetylneuraminyl-N-acetyllactosamine. The nomenclature, standard graphical representation and the computational characterization of the oligosaccharide are also presented*

interaction could explain the bi-directional signaling with the ganglioside and the cis homodimerization observed through the domains Ig4 and Ig5. (Pronker et al., 2016) Like other Siglecs, MAG is an endocytic receptor : via a clathrin-dependent mechanism, it is recycled in the membrane contributing to its modeling. (Winterstein et al., 2008)

The full extracellular portion of MAG has been crystallized. The co-crystallization of MAG with 3'-N-Acetylneuraminyl-N-acetyllactosamine helped understand the main interactions with the ligands (Figure 9). As for other Siglecs, the conservative Arg118 forms a salt bridge with the carboxylate of the sialic acid. A hydrogen bonds network involves the hydroxyl group in positions 4, 8, 9 and the NH in 5 with the surrounding Tyr65, Thr128, Tyr124, and Asn126. Position 9 of the sialic acid is close to a hydrophobic pocket formed by the sidechains of Pro64 and Tyr65. (Pronker et al., 2016)

Small molecules have been synthesized to target MAG and possibly better understand its biological function. (Schwardt et al., 2015) In the B. Ernst group, starting from the minimal binding epitope Neu5Acα(2-3)-Gal-β(1-3)-GalNAc, contained in the natural gangliosides GD1a and GT1b, different strategies contributed to identifying high-affinity MAG antagonists (Figure 10).

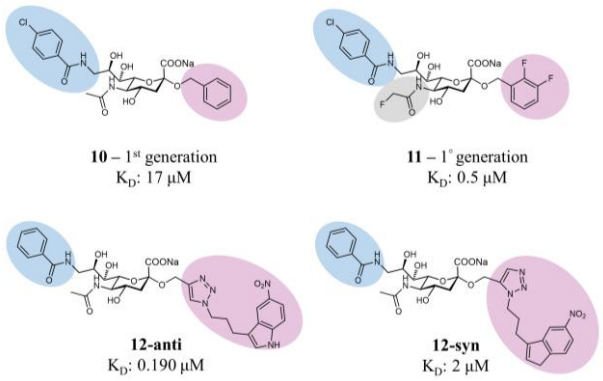


Figure 10. *High-affinity ligands for MAG. Substituents introduced in positions 2, 5 and 9 of the sialic acid are highlighted in pink, grey and blue, respectively.*

In the first two generations of ligands, different modifications were introduced in positions 2, 5 and 9 : in particular, a difluorobenzyl substituent at the 2-position, fluoroacetate at the 5-position, and a p-chlorobenzamide at the 9-position (compounds 10 and 11, Figure 10). These beneficial modifications led to a ligand with nanomolar affinity and drug-like properties.(Mesch et al., 2010 ; Shelke et al., 2007) Later, a combinatorial fragment-based drug discovery strategy identified fragments that could fit into the binding pocket around the sialic acid. The screening identified the 5-nitroindole group, which was subsequently attached to position 9 by click chemistry, leading to high-affinity ligands 12-syn (2 µM) and 12-anti (0.19 µM), as determined by surface plasmon resonance (SPR). (Shelke et al., 2010)

SIGLEC-5 AND SIGLEC-14

Siglec-5 and Siglec-14 are two members of the Siglecs family pair receptors. They occur on the same cells, playing different roles : while Siglec-5 is an inhibitory receptor, Siglec-14 is an activator. Similar to the other Siglecs, the inhibitory activity is associated with the ITIM motif in the intracellular region. Siglec-14 is paired with the activating receptor DAP. Their extracellular domains have the same amino acid sequence in the first two Ig-like domains, showing preferences for the same ligands.(Angata et al., 2006 ; Cornish et al., 1998) Siglec-5 and Siglec-14 occur on neutrophils, macrophages and monocytes. (Lock et al., 2004)

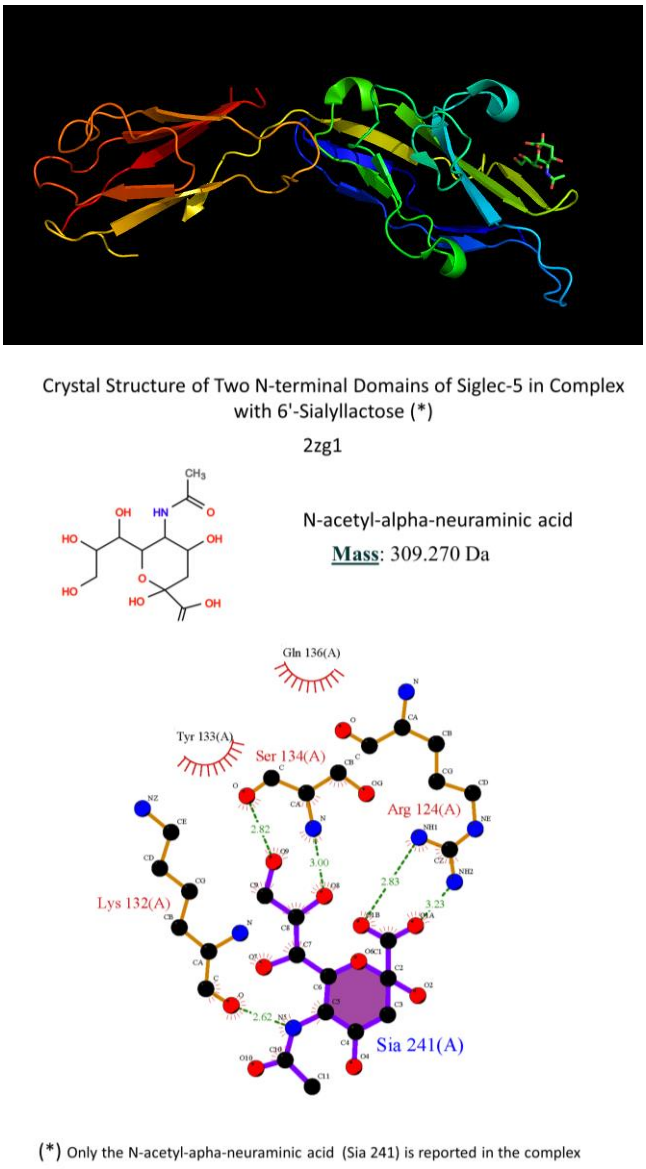


Figure 11. *(Top) Crystal structure of two N-terminal domains of Siglec-5 with 6'-sialyl lactose. PDB code : 2ZG1. (Bottom) Main interactions between Siglec-5 and Neu5Ac (part of the 6'-sialyllactose ligand).*

Some pathogens can exploit Siglec-5 to escape the immune response. For example, group B Streptococcus (GBP) uses a membrane-anchored β-protein to bind Siglec-5 that, being an inhibitory receptor, causes the inactivation of the immune cells. (Carlin et al., 2009) The pairing to Siglec-14 and its activating activity can trigger the immune response. From an evolutionary perspective, the development of a pairing mechanism may have been a response to pathogens. (Ali et al., 2014) Siglec-14 can also interact with *H. influenza* in a sialic acid-dependent manner, stimulating the proinflammatory activity of macrophages, exacerbating the inflammation states, and

increasing the chances to cause chronic obstructive pulmonary disease (COPD). (Chang & Nizet, 2014) The V-set and C2-type Ig domains of Siglec-5 have been crystallized in complex with two sialylated carbohydrates, the α2-3- and α2-6-sialyl lactose (Figure 11). The interactions are quite weak, in the low mM range.

The main interactions involve a salt bridge between the carboxylate of the sialic acid and the conserved Arg124, hydrogen bonds between Lys132 and the amide in position 5 and between Ser134 and the hydroxyls in position 8 and 9. Fewer contacts involve the lactose part of the ligand. (Movsisyan & Macauley, 2020 ; Zhuravleva et al., 2008)

A few Siglec-5/Siglec-14 ligands have been identified, but the affinity of none of them was determined. Modifications were introduced in position 5 or 9 of the sialic acid. The binding to Siglec-5 or Siglec-5/Siglec-14 was evaluated in two ways : by glycan microarray or by metabolic incorporation in cells. In this last case, the ligands underwent further derivatization by click chemistry ; the binding was analyzed by flow cytometry. (Büll et al., 2017 ; Rillahan et al., 2012)

SIGLEC-6

Siglec-6 is a member of the Siglecs family expressed on mast cells, B cells, and, notably, on placental trophoblasts, where it may slow down the human birth process. The expression on these last cells is human-specific. (Brinkman-Van Der linden et al., 2007) Siglec-6 contains a V-set domain, two C2-set domains, and one ITIM domain in the cytoplasmatic tail, thus contributing to negative signallng. (Crocker et al., 2007 ; Patel et al., 1999)

Little is known about the role of this protein.

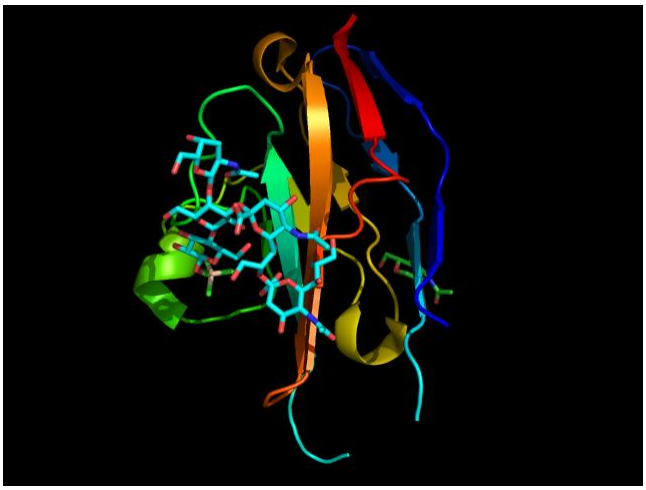
Recent evidence showed a higher expression of Siglec-6 in circulating and urinary T cells in patients who have non-muscle-invasive bladder cancer (NMIBC), a disease associated with high mortality and morbidity. The higher expression was also associated with lower survival. These data indicate Siglec-6 as a possible target for treating this kind of cancer. However, such preliminary findings require further studies to confirm its role in regulating CD8+ T-cell function and bladder tumor immune escape leading to tumor progression. (Benmerzoug et al., 2021) Siglec-6 is also considered a possible target for immunotherapy in chronic lymphocytic leukemia (CLL). (Kovalovsky et al., 2021) Finally, overexpression of Siglec-6 has been detected in preterm preeclampsia placenta, characterized by placental abnormalities ; however, its role in the disease is not clear yet. (Rumer et al., 2013)

Regarding its affinity, it is a protein able to bind leptin, but no other ligands are known. Besides, no solved structure is available.

SIGLEC-7

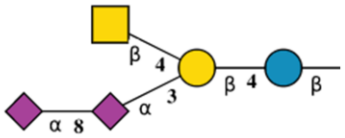
Siglec-7 (also known as p75/AIRM1 or CD328) is a human CD33-related Siglec, described as an inhibitory receptor primarily expressed on natural killer (NK) cells and present on monocytes, dendritic cells, granulocytes and some CD8+ T cells.(Gieseke et al., 2012 ; Maggi & Moretta, 2020 ; Shao et al., 2016) It shares high sequence homology with Siglec-9. (Fraschilla & Pillai, 2017) Siglec-7 has three Ig-like extracellular domains (one N-terminal V-set and two C2-set Ig domains) and a cytoplasmic tail containing one ITIM and one ITIM-like domains, involved in the inhibitory signal inside the cell. (Maggi & Moretta, 2020 ; Yamakawa et al., 2020)

Tumor cells exploit the negative signaling initiated by Siglec-7 : overexpressing Siglec-7 ligands, these cells evade evade the immune system, blocking the NK cell-mediated lysis.(Maggi & Moretta, 2020) For this reason, Siglec-7 has gained attention as a potential target for the treatment of cancer.



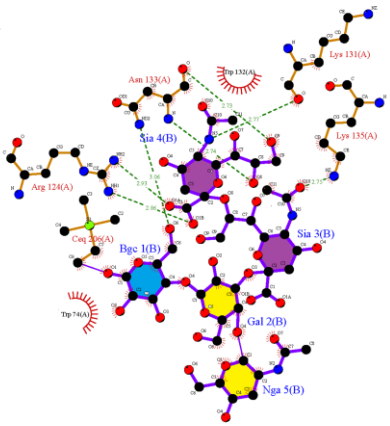
Siglec-7 in complex with GT1b

2hrl



Mass: 1127.999 Da

DNeup5Acα2-8DNeup5Acα2-3[DGalpNAcβ1-4]DGalpβ1-4DGlcpb1-ROH



N-acetyl-alpha-neuraminic acid-(2-8)-N-acetyl-alpha-neuraminic acid-(2-3)-[2-acetamido-2-deoxy-beta-D-galactopyranose-(1-4)] beta-D-galactopyranose-(1-4)-beta-D-glucopyranose

WURCS=2.0/4,5,4/[a2122h-1b_1-5][a2112h-1b_1-5][Aad21122h-2a_2_6_5*NCC/3=O][a2112h-1b_1-5_2*NCC/3=O]/1-2-3-3-4/a4-b1_b3-c2_b4-e1_c8-d2

Figure 12. *(Top) Crystal structure of Siglec-7 in complex with GT1b. PDB code : 2HRL. (Bottom) Main interactions between Siglec-7 and GT1b. The nomenclature, standard graphical representation and the computational characterization of the oligosaccharide are also presented.*

Some studies showed that anti-Siglec-7 mAb inhibits the proliferation of chronic myeloid leukemia (CML) cells *in vitro* and the growth of mast cells *in vitro* and mice, confirming its potential for cancer treatment and diseases linked to mast cells, such as mastocytosis. (Landolina et al., 2020) Next to immune cells, Siglec-7 occurs on plate-

lets, where it causes their apoptosis. (Nguyen et al., 2014) Siglec-7 occur on β-cells of the pancreas, too : the overexpression on these cells leads to a reduction of β-cells dysfunction. It is downregulated in type 1 and 2 diabetes. (Läubli & Varki, 2020)

Siglec-7 preferentially binds to α2-8-linked di-sialylated carbohydrate structures, but it can also bind to α2-6- and α2-3-linked sialic acid-containing glycans. (Gieseke et al., 2012 ; Legrand et al., 2019 ; Movsisyan & Macauley, 2020) Among the six crystal structures of Siglec-7, one has been co-crystallized with the ganglioside GT1b (Figure 12). (Attrill et al., 2006)

The main interactions involve a salt bridge between the carboxylic acid of the sialic acid and the conserved Arg124, three hydrogen bonds between the hydroxyl groups in positions 5, 8, and 9 and the two amino acids Lys131 and Asn133. Hydrophobic interactions involve glucose and the two amino acids Asn133 and Trp74. (Movsisyan & Macauley, 2020)

Serendipity identified one of the first selective ligands for Siglec-7, but no affinity values are available (Figure 13). Compound 13 presents a xanthene group in position 9 of the sialic acid, which confers selectivity to Siglec-7 by forming π-stacking interactions with the cleft close to position 9. (Rillahan et al., 2013)

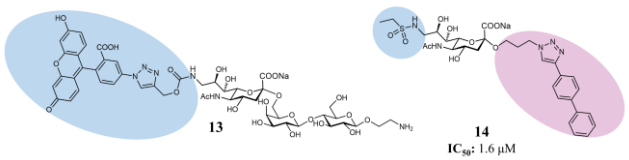


Figure 13. *Chemical structure of Siglec-7 ligands. Substituents introduced in positions 2 and 9 of the sialic acid are highlighted pink and blue.*

One of the most promising ligands has been developed starting from the methyl-α-Neu5Ac. The introduction of a sulfonamide group in position 9 and an aromatic aglycone in position 2 of the sialic acid increased the affinity of 5000-fold compared to the unmodified reference compound (1.6 µM vs 8000 µM ; compound 14, Figure 13). (Prescher et al., 2017)

Further steps towards targeting Siglec-7 have already been made by developing Siglec-7-derived CAR T-cells. They can kill cancer cells by binding to carbohydrates, with a recently found *in vitro* efficacy. (Lenza et al., 2020)

SIGLEC-8

Siglec-8 was discovered around 2000 by two groups independently and identified as a Siglec protein exclusively expressed on eosinophils and mast cells and weakly on basophils.(Floyd et al., 2000 ; Kikly et al., 2000) Siglec-8 consists of a V-set domain, two C-2 set domains, one ITIM and one ITIM-like domains in the intracellular region. It is involved in negative cell signaling. Siglec-8 cross-linking induces apoptosis in eosinophils, in a caspase-dependent manner on normal cells, and through the production of reactive oxygen species (ROS) in interleukin-5 (IL-5) primed eosinophils. (Nutku-bilir et al., 2008 ; Nutku et al., 2003) On mast cells, Siglec-8 inhibits their degranulation, but it does not affect their survival. (Yokoi et al., 2008) Due to the selectivity of expression, the inhibition activity that Siglec-8 has on eosinophils and mast cells, and the constant levels of expression on cells both in healthy and non-healthy conditions, in the last years, much interest has emerged to target this protein for the treatment of many allergic and inflammatory diseases associated with eosinophils and mast cells.

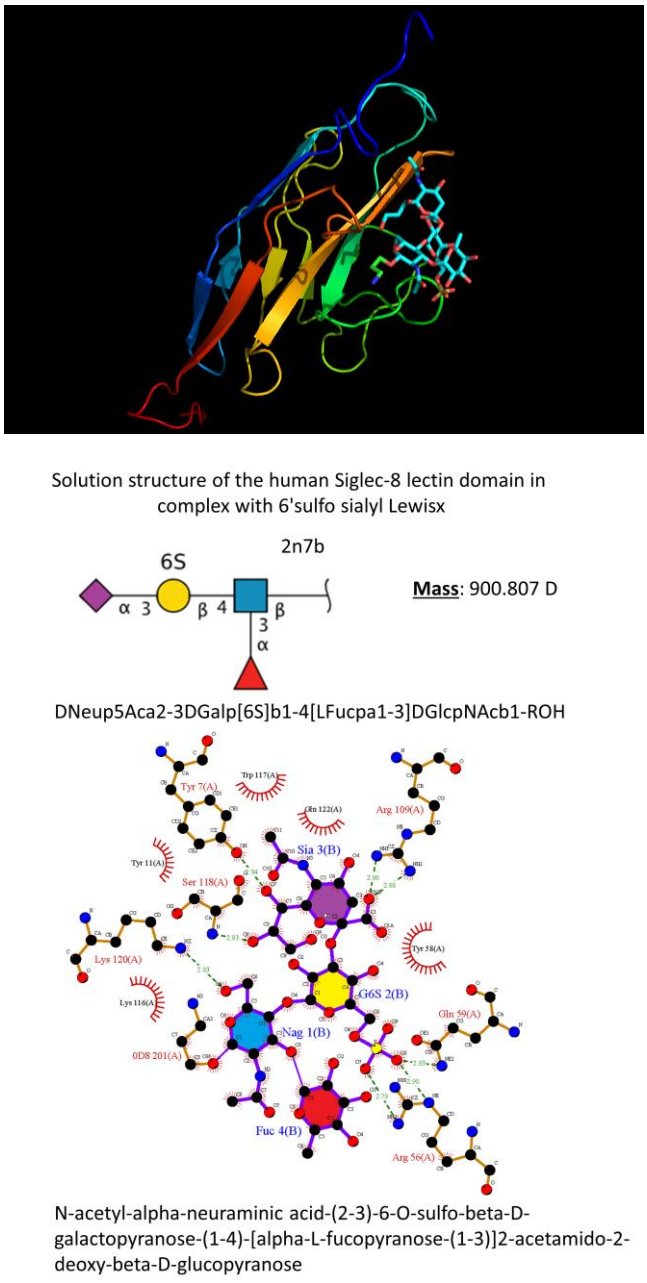


Figure 14. *(Top) NMR solution structure of Siglec-8 lectin domain together with 6'-sulfo-sLe^x. PDB code : 2N7B. (Bottom) Main interactions between Siglec-8 and 6'-sulfo-sLex. The nomenclature, standard graphical representation and the computational characterization of the oligosaccharide are also presented.*

Different results have already validated its potential as a target. AK002 (lirentelimab), for example, is a humanized non-fucosylated IgG1 anti-Siglec-8 antibody already in phase I clinical trials for the treatment of indolent systemic mastocytosis (ISM) and allergic conjunctivitis. The drug improved patients' general quality of life and

relieved comorbidities like asthma, dermatitis, and rhinitis.(Levine et al., 2020 ; Siebenhaar et al., 2019) Encouraging results were reported for phase II trials to treat chronic refractory urticaria (on patients not responding to antihistamines or omalizumab) and eosinophilic gastritis and duodenitis. Long-term use was well tolerated in the last case and showed histological improvements.(Altrichter et al., 2019 ; Dellon et al., 2020)

Anti-Siglec-8 monoclonal antibodies reduced non-allergic inflammation by inhibiting IgE-independent mast cell activation. (Schanin et al., 2021)

Several findings also support the idea of using Siglec-8 in the treatment of asthma. For example, there is an upregulation of Siglec-8 ligands in inflamed human airway tissues compared to healthy tissues, and eosinophils obtained from allergy patients showed increased Siglec-8-mediated apoptosis. (Jia et al., 2015 ; von Gunten et al., 2007) In addition, polymorphisms in the Siglec-8 gene are associated with increased susceptibility to asthma. (Gao et al., 2010) Lastly, it seems that eosinophils and mast cells are also quite active during SARS-CoV-2 infections and that treatment with anti-Siglec-8 antibodies reduces the general inflammation. Hence, upon confirmation by further studies, Siglec-8 may represent a possible target to fight this viral infection causing the actual global pandemic. (Gebremeskel et al., 2021)

As for the other Siglecs, the main interaction involves a salt bridge between the carboxylate of the sialic acid and the conserved arginine residue (Arg109). The essential sulfate in position 6 of the galactose moiety is involved in a second salt bridge with Arg56 and Gln59. In addition, hydrogen bonds exist between hydroxyl groups 7, 8 and 9 of the sialic acid and Tyr7, Ser118 and Gln122. (Pröpster et al., 2016) Considering that the fucose and glucosamine subunits show only minor interactions with the protein, the 6-sulfo-Sia-Gal 16 is likely to be the minimal binding epitope for Siglec-8. Further optimization with a deoxygenation strategy and introducing a sulfonamide substituent in position 9 of the sialic acid led to identifying a potent Siglec-8 ligand (18), with 20-fold affinity improvement compared to the parent tetrasaccharide (Figure 15). (Kroezen et al., 2020 ; Nycholat et al., 2019)

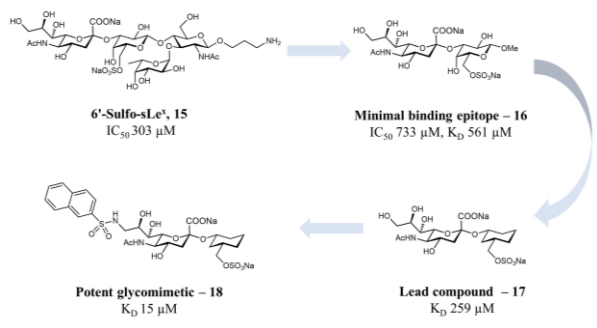


Figure 15. *Development of high affinity Siglec-8 glycomimetic ligands*

The selective expression of Siglec-8 and endocytic property can also be exploited for the selective delivery of therapeutic agents to mast cells and eosinophils to treat malignancies associated with these cells. (O'Sullivan et al., 2018) Next to antibodies, Siglec-8 has also been targeted with nanoparticles displaying its ligands. Liposomes decorated with Siglec-8 ligands were selectively taken up in cells expressing Siglec-8 or Siglec-F (Siglec-8 paralog in mouse). Furthermore, these liposomes could suppress IgE-mediated mast cell degranulation when additionally decorated with allergens.(Duan et al., 2021 ; Nycholat et al., 2019)

SIGLEC-9

Siglec-9 occurs in human blood on neutrophils, monocytes, plasma cells and a subset of NK cells. It is an inhibitory protein, thus presenting in the cytoplasmatic tail the two putative ITIM domains, while on the extracellular portion, the standard V-set domain and two C-2 set domains. (Zhang et al., 2000)

Considering its expression and the inhibitory activity on cells of the immune system, Siglec-9 represents a potential target for cancer, inflammations and viral infections.

Malignant cells usually have a different glycosylation pattern, resulting in correct hyper-sialylated glycans, therefore providing a high number of ligands for Siglecs. (Büll et al., 2014 ; Fuster & Esko, 2005) T-cells and NK cells are both tumor-infiltrating cells, participating in the recognition and killing of cancer cells, and they both express Siglec-7 and Siglec-9. (Daly et al., 2019) Siglec-9 is upregulated in some tumor-infiltrating CD8+ and CD4+ T-cells in various cancers. Studies show that the binding of Siglec-9 with antigen-binding fragments (Fab) of Siglec-9-blocking antibodies increased immune cell activity, reducing the interactions of Siglecs with their ligands. The same effect was reached by treating target cancer cells with neuraminidases, which decrease the availability of sialic acid on the surface and, consequently, the interactions with Siglecs, thus enhancing the immune response. However, the treatment with full-length anti-Siglec antibodies inhibits cell cytotoxicity, suggesting the induction of inhibitory signals.(Haas et al., 2019 ; Hudak et al., 2014 ; Jandus et al., 2014 ; Nicoll et al., 2003 ; Stanczak et al., 2018) A better comprehension of Siglec-9 signal regulation is therefore needed.

A recent study shows how blocking Siglec-9 interactions with sialylated sugars on NK cells increased their cytotoxicity toward HIV-infected cells. (Adeniji et al., 2021)

Another trick that pathogens use to escape the immune system is to mimic self sialic acid-containing sugars. In this way, Group B Streptococcus (GBP) binds to Siglec-9 expressed on neutrophils. As an inhibitory receptor, this leads to the deactivation of the immune response and the bactericidal activity. (Chang & Nizet, 2020) Siglec-9 can also be a target for treating other diseases associated with neutrophils, such as asthma, chronic obstructive pulmonary disease, and pulmonary disorders related to neutrophilia. Recent evidence also suggests a role of neutrophils in the inflammation due to Covid infection. The binding and clustering of Siglec-9 on the cell surface of neutrophils with monoclonal antibodies or polyvalent sialoglycan ligands induce death in neutrophils, significantly when activated. (Chen et al., 2018 ; Delaveris et al., 2021 ; Von Gunten et al., 2005)

Siglec-9 shows high sequence homology with Siglec-7, but despite this, Siglec-9 prefers the binding to α2-6 and α2-3-sialyl residues, while Siglec-7 prefers α2-8 linkages. (Varki & Angata, 2006) In particular, a glycan microarray showed a preference for sialyl Lewis^x, or the same tetrasaccharide with a sulfate group on the GlcNac moiety. (Yu et al., 2017)

Up to date, the Siglec-9 structure remains unknown.

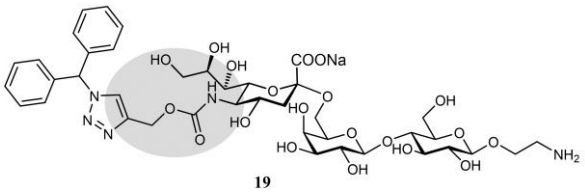


Figure 16. *Selective Siglec-9 ligand identified by glycan microarray.*

As for other Siglecs, glycan micro-arrays lead to identifying a selective Siglec-9 ligand (Figure 16). No affinity was measured, but liposomes displaying ligand 19 selectively targeted Siglec-9+ peripheral blood mononuclear cells. (Rillahan et al., 2012)

SIGLEC-10

Siglec-10 is an inhibitory receptor expressed on B cells, dendritic cells and leukocytes. Its structure comprises five extracellular domains, two tyrosine-based motifs in its cytoplasmic tail and one Grb2 binding motif close to the membrane.(Duan & Paulson, 2020 ; Li et al., 2001)

Recent studies showed how tumor-associated macrophages present high levels of Siglec-10, which interacts with CD24, a sialoglycoprotein expressed on tumor cells. This interaction promotes immune evasion. Genetic ablation of either CD24 or Siglec-10, or the blockage of the interaction using monoclonal antibodies, increase the phagocytosis of all CD24-expressing human tumor cells. (Barkal et al., 2019)

Siglec-10 recognizes α2-3- or α2,6-linked sialoglycans in the micromolar range. Although the crystal structure of Siglec-10 is not available, a recent work that combines NMR, docking and molecular modeling gives some insights into the molecular recognition and binding process mechanism that drive Siglec-10-ligands interactions. (Forgione et al., 2020)

The only known ligand for Siglec-10 is compound 20 (Figure 17), which contains a triazole-linked adamantane group in position 9 of the sialic acid. The same ligand displayed on liposomes bound Siglec-10+ human peripheral blood cells. (Rillahan et al., 2012)

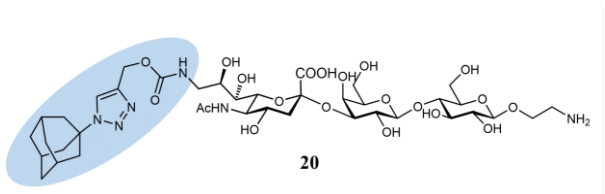


Figure 17. *Siglec-10 ligand identified by glycan microarray.*

SIGLEC-11 & SIGLEC-16

As with Siglec-5 and Siglec-14, Siglec-11 and Siglec-16 form a pair of receptors with opposing functions : Siglec-11 is an inhibitory receptor while Siglec-16 is an activating one. They are both expressed in tissue macrophages and microglia. The expression of Siglec-11 on brain microglia is specific to humans. (Angata et al., 2002 ; Cao et al., 2008b) The nonfunctional Siglec-16P allele converted human Siglec-11, and the converted Siglec-11 allele became fixed in humans. As a result of this series of gene conversions, the extracellular portion of Siglec-11 and Siglec-16 is very similar. Consequently, they have maintained the same preferences in ligand binding, particularly towards α2-8-linked sialic acid oligomers and polymers (polySia).(Angata et al., 2002 ; Shahraz et al., 2015 ; X. Wang et al., 2012)

The roles in neurological disorders are still not clear. Siglec-11 interacts with polysialic acid in the brain, involved in diseases like schizophrenia, autism, and bipolar disorder. (Siddiqui et al., 2019) Studies revealed a neuroprotective function, reduced microglial phagocytosis of apoptotic neuronal material, and microglial neurotoxicity. (Y. Wang & Neumann, 2010) As a counterpart, the DAP12-associated activating receptor Siglec-16 activates the microglial phagocytic activity and proinflammatory signals. A perfect balance between these two signals is essential to maintain brain tissue homeostasis. It eliminates extracellular aggregates without causing inflammation. An impaired activity could otherwise lead to neuroinflammation and neurodegeneration. (Linnartz et al., 2010)

Structural information about these two proteins and the identification of possible ligands are still missing.

SIGLEC-15

Siglec-15 is a high evolutionary conserved Siglec, characterized in 2007. (Angata et al., 2007) It exclusively occurs on myeloid cells and osteoclasts. Structurally, it only presents one V-set domain and one C2-set domain, while in the intracellular region is coupled, through the positively charged Lys274, with the activating co-receptors DAP12 and DAP10. Unlike the other activating Siglecs, it does not have an inhibitory counterpart.

Siglec-15 stimulates bone absorption and osteoclasts differentiation on osteoclasts, as confirmed by Siglec-15 targeting with monoclonal antibodies. These findings suggest that targeting this protein could be a successful therapeutic strategy for treating diseases associated with bone loss, like osteoporosis. The use of an anti-Siglec-15 antibody in mice showed potent osteoclastogenesis inhibition, bone resorption suppression, and an overall increase of bone strength. (Tsuda et al., 2022) Other studies showed how a Siglec-15-neutralizing antibody also stimulates bone formation in estrogen deficiency-induced osteoporosis and promotes ossification at cortical bone's damaged area in fracture healing mouse models. (Zhen et al., 2021)

Siglec-15 also plays a role in the tumor microenvironment and it is expressed on tumor-associated macrophages. Some results showed that Siglec-15 suppresses T cell proliferation, essential in regulating tumor growth. (J. Wang et al., 2019) Cancer growth upon targeting Siglec-15 with a monoclonal antibody in wild-type mice was limited : in this disease model, it seems that Siglec-15 acts as a ligand for an unknown receptor expressed on T cells. Whether the interactions with sialic acids and Siglec-15 are involved in this process is still unknown. (Angata, 2020) A humanized anti-Siglec-15 mAb, the NC318, will soon start the phase II clinical trial to treat advanced solid tumors. In phase I, it was well tolerated, and it showed prolonged stabilization of the disease. (Angata, 2020)

The preferred ligand of Siglec-15 is the Sialyl-Tn (Neu5Acα2-6GalNAc). (Angata et al., 2007) A cell-based glycan array helped identify other possible ligands. Cells lacking sialic acids on the surface were functionalized by introducing alkyne-modified sialic acid on cell surface glycoconjugates by sialyltransferase. Click chemistry reactions were then employed to derivatize the alkyne group with different types of azides. Siglec-15 binds to α2-6-sialosides but also α2-3-linked sialosides. (Briard et al., 2018) Ligand 21 shown in the figure below, was identified as the best ligand of this series (Figure 18)

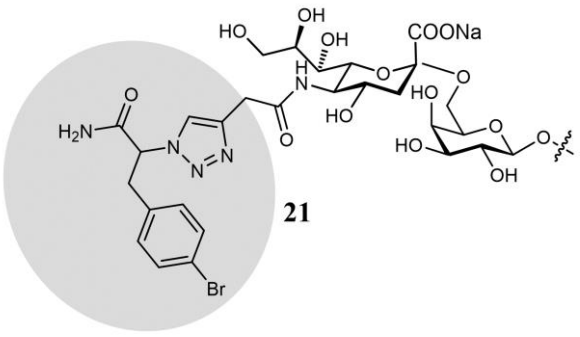


Figure 18. *Siglec-15 ligand.*

Future studies that may better reveal the mechanism involved in Siglec-15/T-cells interactions and the identification of potent inhibitors could open the way to new therapeutic options for cancer and diseases associated with bone loss.

CONCLUSIONS

The idea behind this chapter was to give a general and comprehensive overview of the most recent discoveries about Siglecs, a family of proteins that emerged in the last years as promising pharmacological targets for many diseases.

The sialic acid, recognized by Siglecs, is the most abundant sugar of the glycocalyx. It is involved in several processes, and it represents one of the first contact points between cells. Therefore, it highlights the enormous potential of modulating Siglec-sialic acid interactions. Different strategies have been deployed, from the use of antibodies, which in some cases are already in clinical trials, to the development of small molecules, particularly challenging because of the poor drug-like properties of the natural carbohydrates ligands. Despite all the progress made in the last years, much is yet to be understood. Hopefully, this chapter will contribute to intriguing future glycoscientists about this promising topic, to learn about Siglecs' biological roles and find new ways to address diseases related to autoimmunity, infections, inflammation, ageing, and cancer.

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