Substantial Basis for Glyco-Assembly: Siglec7 and Synthetic Sialylpolymers

Sayo Morishita, Masaya Hane, Ken Kitajima, and Chihiro Sato

Abstract Sialic acid is one of the tags that are recognized as "self," when immune cells survey "non-self" items using the sialic acid-recognizing lectins, Siglecs, on their cell surface. These self-non-self discriminating machineries give not only an advantage to the host cells in innate immune system but also a disadvantage because they allow cancer cells to escape from the immune cells. Siglec7 is one of such machineries predominantly expressed in natural killer (NK) cells and acts as an inhibitory receptor in a sialic acid-dependent manner; however, how Siglec7 binds the sialic acid residues on its counter-receptors has still remained unknown. Since 1999, many reports have described the binding properties of Siglec7 using various sialic acid-containing materials, mostly focusing on the sialyl linkage and the types of core glycan structure. Very recently, two new features of Siglecs binding have been reported: One is the discovery of the new binding sites in various Siglec molecules different than the conventional binding site; the other is the finding of the mucin-type ligands as a natural counter-receptor of Siglec7. These new features thus led us to overview the new binding sites of Siglec7, structural aspects and affinity for the SIglec7 ligands, and natural ligands. In this chapter, we describe the synthetic and natural ligands for Siglec7 and discuss their ligand-binding properties, which are important for therapeutic drug development especially under the spot light of multivalency.

S. Morishita
Graduate School of Bioagricultural Sciences, Nagoya University, Chikusa, Nagoya, Japan

Bioscience and Biotechnology Center, Nagoya University, Chikusa, Nagoya, Japan

M. Hane · K. Kitajima · C. Sato (✉)
Graduate School of Bioagricultural Sciences, Nagoya University, Chikusa, Nagoya, Japan

Bioscience and Biotechnology Center, Nagoya University, Chikusa, Nagoya, Japan

Integrated Glyco-BioMedical Research Center (iGMED), Institute for Glycocore Research (iGCORE), Nagoya University, Chikusa, Nagoya, Japan
e-mail: chi@agr.nagoya-u.ac.jp

K. Furukawa, M. Fukuda (eds.), *Glycosignals in Cancer*,
https://doi.org/10.1007/978-981-19-7732-9_3

1 Introduction

Siglecs are sialic acid (Sia)-binding immunoglobulin (Ig)-like lectins (Crocker et al. 2007) that comprise a Sia-binding N-terminal V-set domain, 1–16 C2-set Ig domains, a transmembrane domain, and a cytosolic region. The V-set domain is important for the interaction of Sia with the immune system (Fig. 1). The crystal structure of Siglec with a Sia analogue demonstrates the critical Sia binding at the Arg (R) residue on the F strand of Siglec; all Siglecs except for Siglec12 have this essential R residue (Alphey et al. 2003; Crocker et al. 2007) (Fig. 2, 1O7V, 2HRL). The majority of Siglecs are predominantly expressed in immune cells, and their expression is cell type-specific. The cytosolic regions of almost all Siglecs have an immunoreceptor tyrosine-based inhibition motif (ITIM) and/or an ITIM-like motif

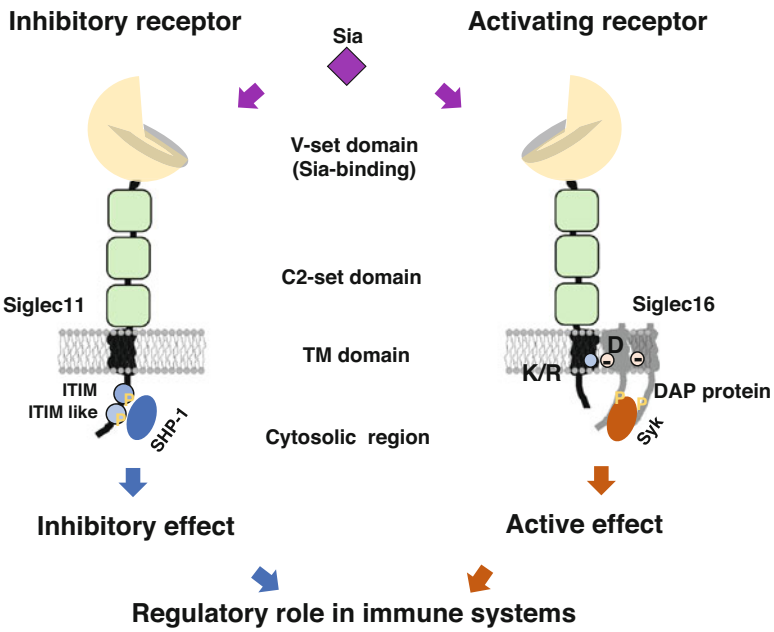

Fig. 1 Schematic structure of Siglec. Siglecs are categorized into two groups based on their functions as follows: an inhibitory receptor and an activating receptor. Extracellular domain of Siglecs is common. All Siglecs have one V-set domain, 1–16 C2-set domains, a transmembrane domain, and a cytosolic region. The V-set domain is the critical region for Sia (Neu5Ac) (ligand, purple diamond) binding and is involved in distinguishing between self and non-self. Inhibitory Siglec has an ITIM or/and ITIM-like motif where SHP-1 is recruited via phosphorylation (P, tyrosine residues) of both motifs. Inhibitory receptor suppresses the immune response to regulate the signal. Activating receptor has an R or K residue in the transmembrane domain, and DAP is bound to Siglec via a D residue in its transmembrane domain. DAP recruits Syk and activates the immune response. At times, Siglecs shows paired receptors, as observed in case of Siglec11 (for inhibitory receptor) and Siglec16 (for activating receptor). The V-set domains of Siglec11 and Siglec16 are the same; therefore, the binding property toward Sia is the same, but the immune response is reverse

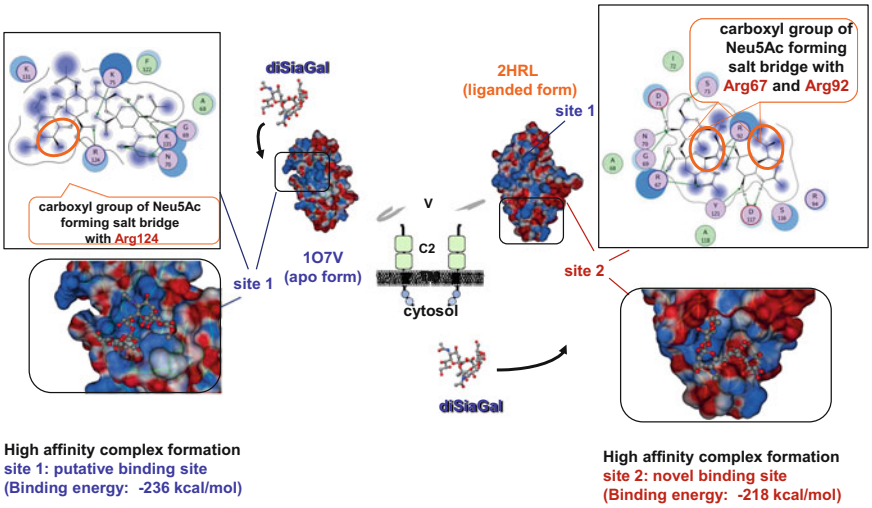

Fig. 2 Schematic structure of Siglec. Molecular modeling of Siglec7. Crystal information of apo-Siglec7 (1O7V) and the ligand form of Siglec7 (2HRL) was prepared. After removal of GT1b analogue ligand information from that of 2HRL, both structures were docked with diSia structure. The highest energy models are shown. Left, 1O7V with diSia structure. Right, 2HRL with diSia structure. The 1O7V residue showed docking with the primary Sia-binding site, and the carboxyl group of Neu5Ac formed a salt bridge with R124. Siglec7 model calculated using 2HRL as a template showed the docking with the secondary Sia-binding site; the carboxyl groups of Neu5Ac formed salt bridges with R67 and R92. In in vitro experiments, R67 but not R92 was shown to be involved in Sia binding. R92 is somehow related to ligand binding. Interestingly, R94 was later shown to be the common amino acid among all Siglecs (see Fig. 3). Considering the location, site 1, primary Sia-binding site, might be related to the trans-ligand, and site 2 or the secondary Sia-binding site might be related to the cis-ligand

(s) to regulate signal inhibition; therefore, such Siglecs are inhibitory receptors that recruit signaling molecules such as SHP1 via ITIM and ITIM-like regions. There are several paired Siglecs such as Siglec11 and Siglec16 where one acts as an inhibitory receptor and the other serves as an activating receptor (Fig. 1). The extracellular domains of these paired Siglecs are identical. The striking difference in the activating receptor is the transmembrane and cytosolic region, especially the presence of an Arg residue within the transmembrane region that mediates the recruitment of the adaptor protein and DAP to regulate signal activity via signaling modules such as Syk (Fig. 1). Siglecs are important for immune cell functions via Sia binding, and any disturbance in the interactions between Sia and Siglec alters the state of the immune system and may lead to infection, inflammation, autoimmunity, and cancer-escaping system (Crocker et al. 2007). In general, Siglecs are considered to be receptors for distinguishing self from non-self; however, the precise mechanism of action of the "self"-"non-self" distinguishing system, natural ligands, molecular basis of the catch and release of ligands, and recycling remains unclear.

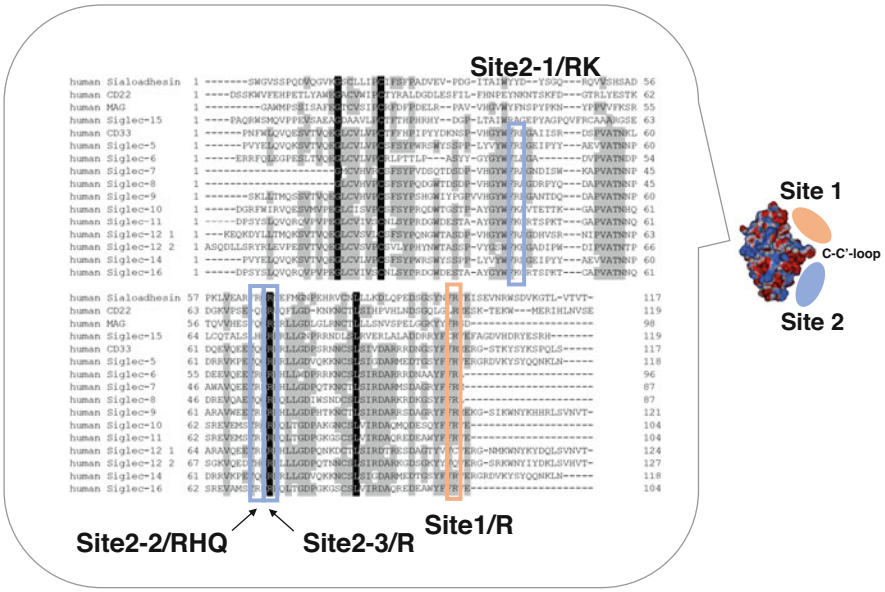

Fig. 3 Amino acid sequence of Siglec. Amino acid sequences of human Siglec1–16 were aligned. The common amino acid sequences among all Siglecs are shown in black. Site 1 is shown in pink and site 2 in blue. Right panel shows the crystal structure of Siglec7. The Siglec12, that is considered to have no Sia-binding ability, has no R in site1

As Siglecs are important for the immune system and cancer-escaping system, the understanding of the role of ligands and ligand-binding systems of Siglecs in biological processes or disease pathogenesis is very important. Recent research on Sia-based chemistry has focused on the establishment of biochemical probes with high affinity for Siglecs as well as specific probes for clinical use (Büll et al. 2016; Hudak et al. 2014; Ohira et al. 2017; Prescher et al. 2017; Yamaguchi et al. 2017) because ligands or ligand-binding systems are keys for medicinal targets.

Siglec7 (CDw328), a member of human CD33-related Siglecs, comprises an extracellular N-terminal V-set domain, two C2-type Ig repeat domains, transmembrane domain, an ITIM, and an ITIM-like motif in the cytosolic region (Fig. 2) (Angata and Varki 2000; Nicoll et al. 1999). It is well known that Siglec7 localizes to NK cells and monocytes and functions as an inhibitory receptor. The expression of Siglec7 is downregulated on an NK cell subset ($CD56^{bright}$) in obese humans (Rosenstock et al. 2017). Downregulation of Siglec7 expression during infection with hepatitis C virus leads to dysfunctional NK cell phenotypes, reduced degranulation, and cytokine secretion (Varchetta et al. 2016). Decreased Siglec7 level is also observed in patients with human immunodeficiency virus (HIV) infection (Brunetta et al. 2009). Therefore, Siglec7-expressing NK cells are more functional than Siglec7-negative NK cells and show higher expression of several activation markers and increased cytokine production (Shao et al. 2016). In monocytes, Siglec7 participates in generating a monocyte-mediated inflammatory outcome following

pathogen recognition (Varchetta et al. 2012). In the case of T cells, Siglec7 is expressed on a small subset of $CD8^+/CD3^+$ T cells and Jurkat cells; T cell activation is inhibited via R124 of Siglec7 (Ikehara et al. 2004). Siglec7 has recently been considered to be a target molecule via regulation of Sia-dependent protection of carcinomas from NK cells because cancer cells are protected by the overexpression of Sia on their surfaces from the survey of immune cells. A hypersialylation state on the surface easily helps a cell to escape from NK cells based on the self (Sia)-recognition by Siglec.

Many reports on the function of Siglec7 were appeared as described above; however, there are not so many reports on the understanding the regulatory mechanism of ligands for Siglec7 and on the identification of natural ligands for Siglec7. In this chapter, we described the recent advance on the establishing the synthetic Sia compounds, understating the molecular regulatory mechanism of ligands, and natural ligands.

2 Ligand-Binding Site of Siglec7

Several crystal structures of Siglec7 with or without ligand(s) have been solved so far (Attrill et al. 2006b; Dimasi et al. 2004; Alphey et al. 2003). The highly variable C–C′ loop (residues 70–75 in Siglec7: Fig. 3) of the V-set domain is a key determinant of Siglec specificity, as observed in domain-swapping experiments (Yamaji et al. 2002). The crystal structure of Siglec7 complexed with a synthetic oligosaccharide corresponding to the α2,8-disialylated ganglioside GT1b revealed the involvement of R124 in Sia binding and the marked conformational change in the C–C′ loop (Alphey et al. 2003). Accumulating data from the crystal structure of Siglec7 with or without ligands allow prediction of its ligands using computational analyses. For instance, docking results using two different Siglec7V structures (an apo form, 1O7V, and a liganded form, 2HRL) demonstrated interesting aspects for ligand recognition as follows: first, a new binding site (site 2, region containing R67 and R92) other than the essential binding site (site 1, region containing R124) was suggested; second, an additional glycan group under the diSia epitope influenced the affinity toward ligand; third, the presence of a new binding site may be regulated by ligand interaction; and fourth, the flexible C–C′ loop transition might be the key for stable interaction. Subsequent biochemical analyses confirmed and supported these observations. In particular, in 2020, a new Sia-binding site other than a primary Sia-binding site (site 1, region containing R124) predicted by in silico analysis was shown by in vitro analyses, equilibrium dialysis, and double reciprocal plot using different ligands for Siglec7 (Yamakawa et al. 2020) (Fig. 2). The new binding site was named as site 2, which is a region containing R67 (site 2–1, Fig. 2). Interestingly, another R residue, R94 (site 2–3, Fig. 2), was found to be conserved among all Siglecs (Yoshimura et al. 2021b). R67 and R94 near the edge of the C–C′ loop are thought to regulate this loop. R92 at site 2 (site 2–2, Fig. 2) was also speculated to bind to the ligand for Siglec7; however, no significant changes aside

from slight modifications were observed by in vitro analysis (Yoshimura et al. 2021b). Mutual regulation of sialic acid binding regions, sites 1 and 2, may be the key mechanism underlying the complexity of these ligands.

3 Interaction Between Siglec7 and Synthetic Glycans

Siglec7 was firstly cloned in 1999 from a human primary dendritic cell cDNA library, and its Sia-based binding was confirmed by the interaction between Siglec7-expressing COS-1 cells and erythrocytes (Nicoll et al. 1999). In addition, the authors used biotinylated polyacrylamide glycoconjugates containing Neu5Acα2,3Galβ1,4Glc (2,3-PAA), Neu5Acα2,6Galβ1,4Glc (2,6-PAA), and Galβ1,4Glc (Lac-PAA) (Fig. 4a) and observed stronger binding with 2,6-PAA than with 2,3-PAA; no binding was observed with Lac-PAA. Interestingly, even after sialidase treatment to remove cis-ligands as sialoglycans on COS-1 cells, Siglec-7 showed the same binding property toward 2,6-PAA and upregulated binding toward 2,3-PAA, unlike other Siglecs (Siglec1, CD22, and Siglec5). These phenomena might be attributed to the different binding sites for 2,3Sia and 2,6Sia, as described in Sect. 2 (Fig. 2). In 2000, the same Siglec7 gene was cloned from human peripheral blood mononuclear cells, and the binding specificity was analyzed using a biotin-conjugated polyacrylamide array (Angata and Varki 2000) of Neu5Acα2,6Galβ1,4Glc (2,6Lac-PAA), Neu5Acα2,3Galβ1,4Glc (2,3Lac-PAA), Neu5Acα2,3Galβ1,3GlcNAc (2,3LacNAc(I)-PAA), Neu5Acα2,3Galβ1,4GlcNAc (2,3LacNAc(II)-PAA), Neu5Acα2,3Galβ1,3GalNAc, Neu5Acα2,6GalNAc (2,6Core1-PAA), Neu5Acα2,3Galβ1,3(Fucα1,4)GlcNAc (sLe^x-PAA), and Neu5Acα2,3Galβ1,4(Fucα1,3)GlcNAc (sLe^a-PAA) (Fig. 4a). The highest binding was observed for 2,6Lac-PAA but not for 2,6Core1-PAA, indicating, as described in Sect. 2, that the underlying non-Sia glycan structure or the distance from the backbone polymer structure was also important. In addition, the authors showed the requirement of the glycerol side chain (C_7-C_9) of Sia by periodate treatment. In 2002, ganglioside glycan probes were used for binding analysis (Yamaji et al. 2002). The oligosaccharide part of the ganglioside was released from the native ganglioside by ozonolysis, and the released glycan was conjugated to streptavidin (SA) by reductive amination. The oligosaccharide-SA was then coupled with biotinylated and radioiodinated bovine serum albumin (BSA) to obtain multivalent ganglioside glycans (Fig. 4b). Binding analysis was performed using CHO cells expressing Siglec7, and GD3, GT1b, GD1a, LSTa, LSTb, LSTc, and GM1 probes were prepared. Siglec7 could bind to GD3, GT1b, and LSTb probes (Fig. 4b); it is also bound to GD1a and LSTc, but the binding was only observed for sialidase-treated Siglec7-expressing CHO cells, indicating that the ligand-binding pocket of Siglec7 on CHO cells was already occupied and only opened after sialidase treatment. The presence of different Sia-binding sites, as described in Sect. 2, can be considered. One pocket, probably site 2, may be for a cis-ligand such as GD1a- and LST1c-like molecules. As the GD1a is one of the most abundant gangliosides in the cells, it is

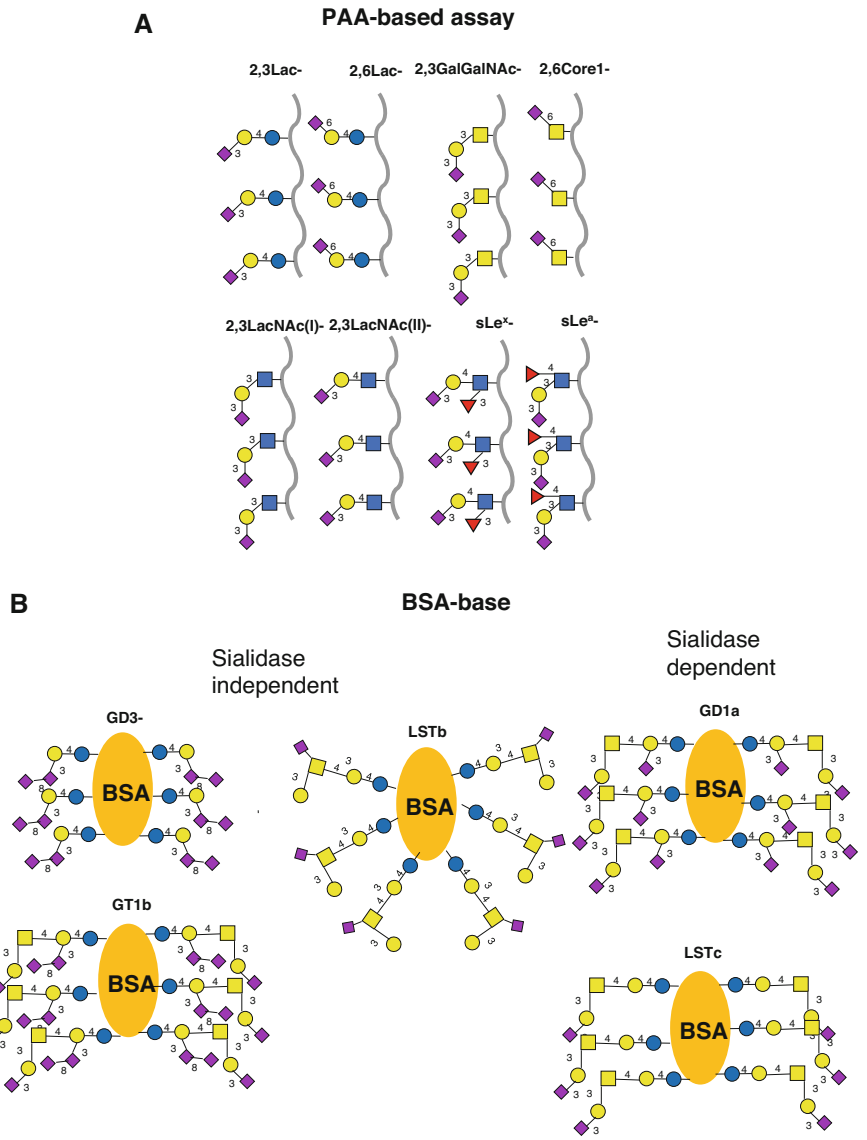

Fig. 4 Siglec ligands used for binding or inhibition assay. (**a**) Polyacrylamide (PAA)-based ligands. 2,3Lac-, 2,6Lac, 2,3GalGalNAc-, 2,6Core1-, 2,3LacNAc(I/II), sLex, and sLea were used, and binding activities were observed. (**b**) BSA-based ligands. Ganglioside oligosaccharides were conjugated to BSA, and multivalent soluble ligands were used for binding assay in Siglec7-expressing cells. GD3, GT1b, and LSTb-BSA probes can bind to Siglec7-expressing cells with any sialidase treatment. On the other hand, GD1a and LSTc showed sialidase-dependent binding. (**c**) ELISA-based ligands. Typical gangliosides were used for the interaction with Siglec7. GD3, GD2, GT1b, GQ1b, GT1a, and 2,6SPG were shown to bind to Siglec7. Disialic, trisialic, and tetrasialic acid containing phosphatidylethanolamine (PE) (Sato et al. 1995) were synthesized and shown to bind to Siglec7. (**d**) Array-based ligands. The glycan array system was used for the analysis of the interaction. Siglec7 showed high activity toward GD3, GD2, triSia, and GT3 and intermediate activity toward Neu5Acα2,6-(Neu5Acα2,3)-GalNAc, 6-sulfosLex, and 6'sulfosLex. (**e**) Ligands used for inhibition. DiSia-octyl, poly-diSia, and diSia-dex were used for the inhibition analysis (see Fig. 5). Only diSia-dex could release Siglec7 from the GD3 plate

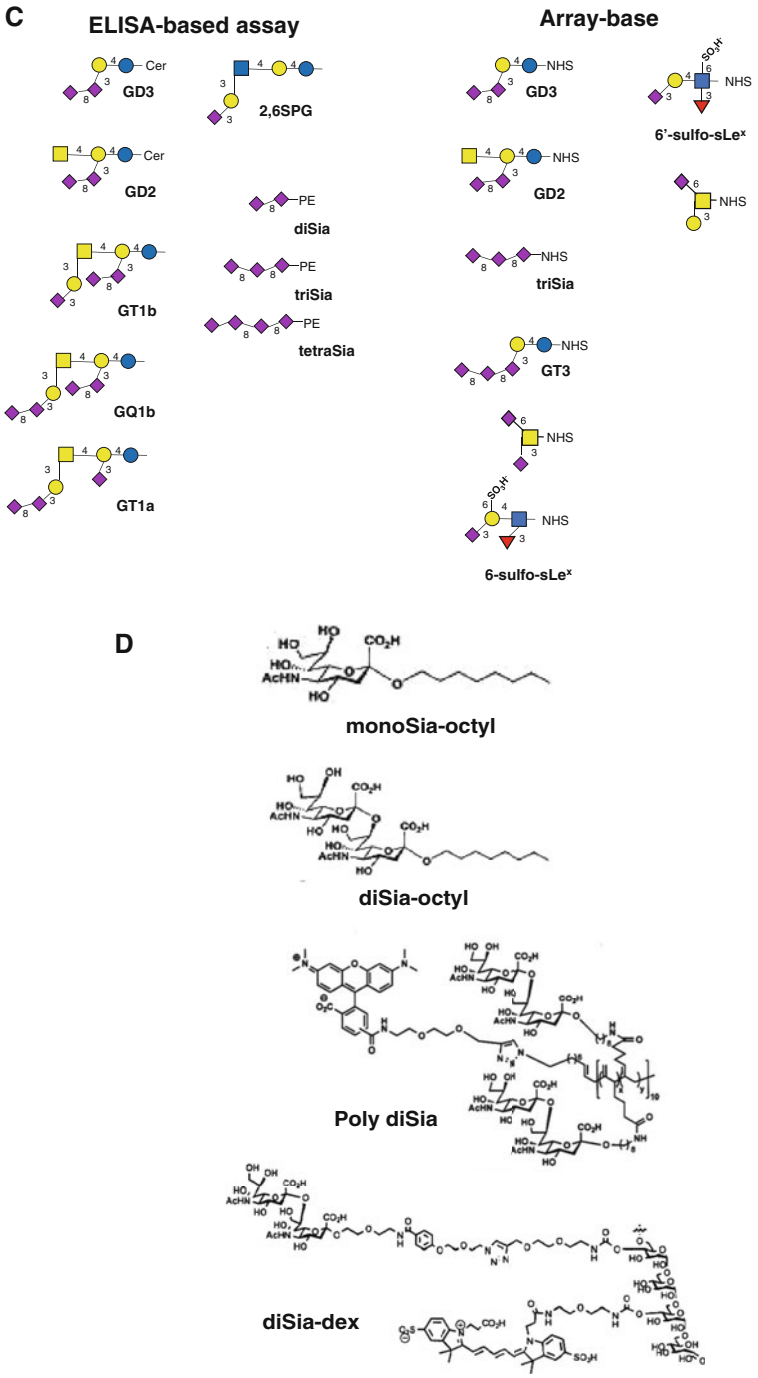

Fig. 4 (continued)

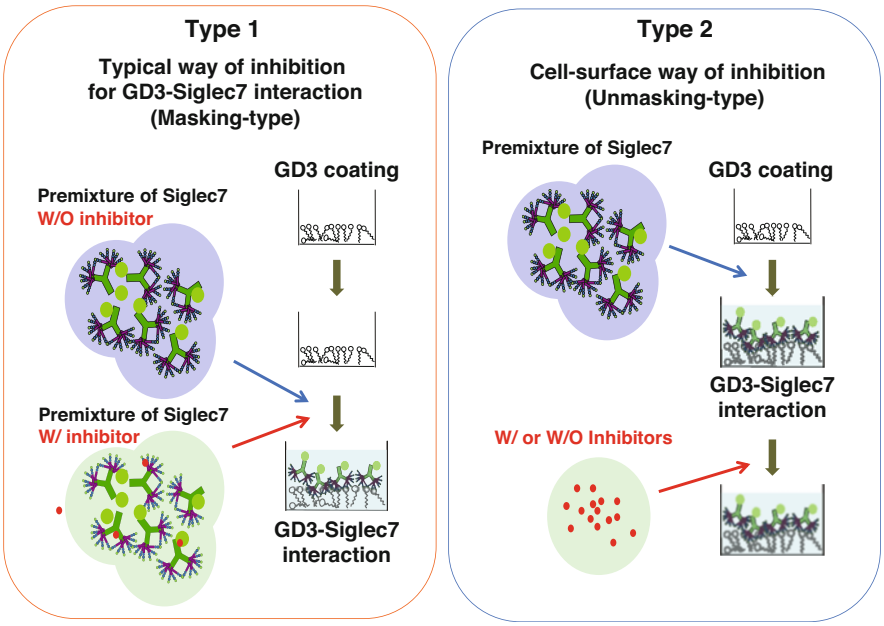

Fig. 5 Inhibition analysis of Siglec7. Two types of inhibition assays were used. Type I inhibition assay is a typical way of inhibition analysis of Siglec7. Type II inhibition assay is a cell surface-type inhibition, which releases the binding site of Siglec7 masked with the ligand on host cells

reasonable that GD1a is the self-signal. Interestingly, Siglec9 did not show any binding toward these multivalent probes, indicating that multivalency is critical feature for Siglec7 binding. In the inhibition assay, free GD3 oligosaccharides inhibited the binding between Siglec7 and gangliosides other than GD1a and LST1c, with IC_{50} values of 5–10 mM. Inhibition analysis using GD3 glycan derivatives such as GD3-C_1 amide, GD3-(C_1-OH), and GD3-(C_7-OH) showed lower inhibition than that observed with GD3 glycan, indicating that C_1 and C_7 positions are important. Chimeric analyses of Siglec7 and Siglec9 revealed a small region (NDISWK) of Siglec7 localized in the C–C′ loop (Fig. 3) responsible for ligand specificity. Interestingly, the three upstream amino acids, R, were shown to form a second Sia-binding site (Figs. 2 and 3) (Yamakawa et al. 2020; Yoshimura et al. 2021b). The binding of GD3, GT1b, and GQ1b was also demonstrated using Siglec7-expressing CHO cells, although the baselines toward these gangliosides were very high (Rapoport et al. 2003) (Fig. 4c).

In 2001, various authentic gangliosides (GD3, GD2, GT1b, GD1a, GD1b, GM2, and GM3), α2,3/6SPG, α2,3-SnLc$_6$, sLe^x, and sLe^a, were analyzed for their interactions with Siglec7. Among these, GD3, GD2, and GT1b appeared to bind Siglec7 (Fig. 4c). In addition, α2,6-SPG could also bind Siglec7 (Ito et al. 2001) (Fig. 4c). Other samples showed no interaction with Siglec7, although GM3 has the same sequence as Neu5Acα2,3Galβ1,4Glc-, which was used to determine the binding using the glycan array system (Nicoll et al. 1999; Angata and Varki 2000). Based on

the solid-phase analysis, the disialyl (diSia) epitope on gangliosides (GD3, GD2, GT1a, GT1b, and GQ1b) was focused on the ligand for Siglec7 (Fig. 4c). Newly synthesized oligoSia-containing glycolipids, oligoSia-PE (Fig. 4c), were analyzed for the requirement of Siglec7 interaction, and di-, tri-, and tetra-Sia containing phosphatidylethanolamine were found to interact with Siglec7 even before sialidase treatment (Yamakawa et al. 2020). The glycan specificity of Siglec7 binding was also analyzed using the glycan array system supplied by the Consortium for Functional Glycomics (Avril et al. 2006a) that showed a new sulfated epitope, (su)-sLe^x (Fig. 4d).

Based on the reports of the interaction between ganglioside glycan and Siglec7, synthetic ganglioside glycans were used to understand the crystal as a ligand-bound conformational form by structural analysis of GT1b and Siglec7 (Attrill et al. 2006a, b). First, oxamide-Neu5Ac was prepared from the methyl α-glycoside of 9-amino-9-deoxy-Neu5Ac via reaction with activated oxamic acid. At the same time, DSLc4 was also used (Attrill et al. 2006b). The synthetic GT1b analog (GT1b-trimethylsilyl)ethyl was used for crystal formation (Attrill et al. 2006a). Both analyses confirmed the interaction between ligands and the R124 residue on Siglec7 that was considered important for Sia binding.

4 Interaction Between Siglec7 and Synthetic Glycopolymers

Glycan multivalency was analyzed using 2,3PAA and 2,6PAA, and almost all results were obtained from multivalent-based ligands or assays (Nicoll et al. 1999) (Fig. 4). The glycan multivalency of Siglecs was also confirmed by liposome-based experiments. The interaction between Siglec1 and its ligand was used as the target of the drug toward macrophages expressing Siglec1. The compound 9-*N*-biphenylcarboxyl-NeuAcα2-3Galβ1-4GlcNAc-ethylamine (39-BPCNeuAc) was introduced into hydroxysuccinimide (NHS)-activated pegylated lipids, and Sia analogue-bearing liposomes were prepared (Chen et al. 2012a). The glycan ligand of Siglec1-decorated liposomes was successfully delivered to macrophages, and internalization was achieved. The same approach was adopted toward CD22 (Chen et al. 2012b). These studies, from the viewpoint of drug delivery, clearly showed that Sia-coated liposomal nanoparticles exhibited effective binding and served as therapeutic material targets for Siglecs. A sialoside library in solution and on-chip was also established (Rillahan et al. 2012, 2013), and a high-affinity ligand for Siglec7 was found where the fluorescein group appended to the C_9 position through a triazole linkage. Researchers also succeeded in synthesizing Siglec7 ligand-coated liposomal nanoparticles to deliver liposomes to Siglec7-expressing cells. The diSia-coated nanoparticles could bind to mouse Siglec-E and inhibit the inflammation of human monocytes and macrophages (Spence et al. 2015).

The consideration of the strength of the affinity of the ligand toward Siglec7 first appeared in the inhibition analysis. The IC_{50} value of the GD3 epitope toward GD3-Siglec7 interaction was 5–10 mM (Yamaji et al. 2002). Later, the synthesized

9-*N*-oxamyl sialosides were shown to have an IC_{50} of 1.6 mM, and the native Neu5Ac had an IC_{50} of 8 mM. If the 9-position was subjected to hydrophobic substitution, the IC_{50} changed to 1/58 (140 μM) of the original value (Prescher et al. 2015). The affinity also increased to 1.6 μM following the substitution at the C-9 position (Prescher et al. 2017). These authors used a monoSia structure for binding monovalent Sia.

The affinity of synthetic diSia structure toward Siglec7 was first measured by Tanaka and Sato (Ohira et al. 2017) who showed that the IC_{50} values of fluorescently labeled poly-diSia (degree of polymerization [DP] = 20), fluorescently labeled poly-diSia (DP = 50), and diSia were 10 μM, 3 μM, and greater than 1 mM, respectively. This was the first quantitative analysis to demonstrate that diSia multivalency effectively changed the affinity. After the development of dextran-based new glycopolymers (Dex), the interaction (K_D) between diSia-Dex and Siglec7 was measured by biolayer interference methods (BLI) and found to be 5.87×10^{-10} M (Yamaguchi et al. 2017). When calculated as the diSia unit, the mono-diSia contribution was almost 2×10^{-7} M. The affinity between diSia-Gal and Siglec7 was 2×10^{-6} M when measured by the equilibrium dialysis method (Yamakawa et al. 2020); based on these values, the multivalent effect on affinity was approximately tenfold. This observation suggests that the multivalency effect was synergistic. The authors also analyzed the inhibitory effect of polySia on the diSia-Siglec7 interaction. PolySia consists of Sia (colominic acid) with a DP of ~40 and could be considered as a polymer of diSia (DP = 20). In addition, the non-reducing terminal ends of polySia and diSia may be the same. However, there was no inhibitory effect of colominic acid on Siglec7-GD3 interaction, indicating that the diSia epitope on polySia, even at the non-reducing terminal end, was not the same glycotope of the diSia structure. This observation is important and consistent with the results of antigen specificity of anti-oligo/polySia antibody toward oligo/polySia structures. In addition, inhibitory effects of glycopolymers such as chondroitin, hyaluronan, heparan sulfate, chondroitin sulfate-6S, chondroitin sulfate-4S, keratan sulfate, and dermatan sulfate were not observed, indicating that any negative charge, except for Sia, is not the cause for interaction in the case of Siglec7. In addition, mannose polymer (mannan), glucose polymer (glucan), and GlcN polymer (chitosan) had no binding ability toward Siglec7.

The IC_{50} of diSia-Dex was shown to be ~1 nM (Yamaguchi et al. 2017), which is almost consistent with the K_D value. The inhibition of monoSia-Dex against binding of GD3 and Siglec7 was achieved at 50 nM concentration, and monoSia-Dex only inhibited half of the binding, indicating that there are several binding sites. As it is demonstrated that Siglec7 had a secondary Sia-binding site around R67, based on in silico analyses, mutation analysis, equilibrium dialysis, saturation transfer difference nuclear magnetic resonance analysis, and reciprocal plots of inhibition assay (Yamakawa et al. 2020), monoSia- and diSia-Dex may distinguish the sites. In addition, each site may show different binding affinity.

For the inhibition analysis, two types of experiments were performed using Siglec7. The typical method (type 1) involves pre-inhibition of Siglec7 using an inhibitor and analysis of GD3 binding (Fig. 5). This inhibition mimicked the

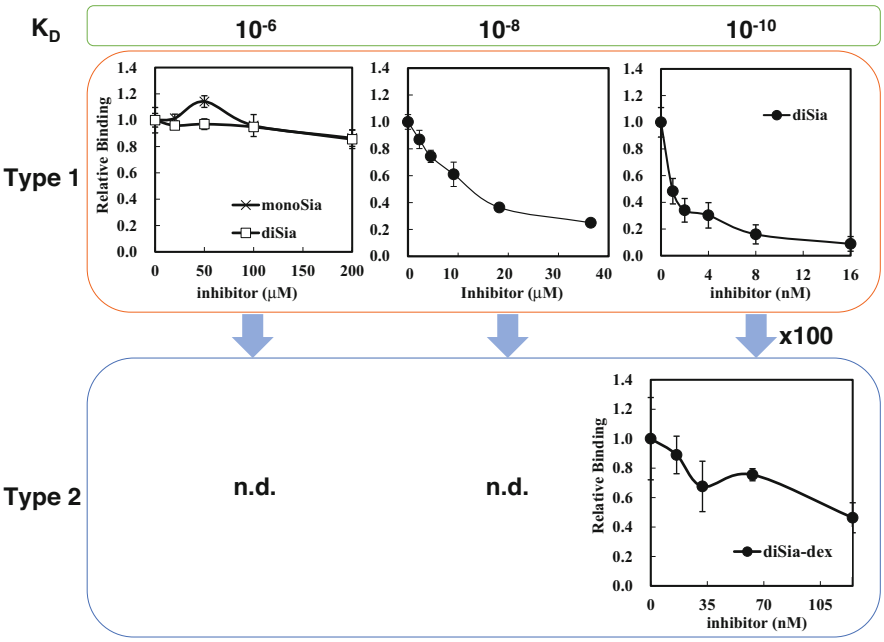

Fig. 6 Summary of the inhibition assay using multivalent ligands. DiSia-octyl, poly-diSia, and diSia-dex were used for the analysis of type I and type II inhibition (see Fig. 5). K_D is also shown. diSia-octyl showed a high K_D value and did not inhibit the interaction between Siglec7 and GD3. Poly-diSia showed only type I inhibition. The K_D is two-order lower than that observed with diSia-octyl. Using diSia-dex, both inhibition types were observed. The K_D was almost 10^{-10} (M), indicating that the affinity greater than 10^{-10} (M) is important for releasing the cis-ligand on host cells from the binding site. Multivalent effect such as mucin-type glycan is necessary

masking effect. In contrast, the type 2 inhibition experiment mimicked the unmasking effect because the inhibitor was used after Siglec7-GD3 binding. These two types of experiments revealed an interesting feature. MonoSia-octyl could not inhibit the interaction (Fig. 6, *upper-left*, monoSia), but 2,3PAA could bind to Siglec7, indicating that the monoSia epitope should be multivalent for Siglec7 binding. This should be a mucin-type O-linked Sia cluster, which was confirmed by thin-layer chromatography (TLC) immunostaining (Yoshimura et al. 2021b). DiSia-octyl also showed no effect on type 1 inhibition (Fig. 6, *upper-left*, diSia), indicating that diSia clusters are required. Unlike GM3, Siglec7 can bind to GD3 ganglioside; thus, the cluster of GD3 localized on lipid rafts may be a ligand candidate. If the diSia epitope increases as a glycopolymer, such as poly-diSia (DP = 20), it shows type 1 inhibition (Fig. 6, *upper-middle*, diSia) and indicates that at least 20 diSia epitopes are required for stable masking. There was no effect on type 2 inhibition using this compound, suggesting that an affinity stronger than that of poly-diSia is required. If the concentration of the diSia epitope in the polymer is increased, as in diSia-Dex, the type I inhibition effect is increased by 100 times. Effective type 2 inhibition was observed using this glycopolymer (Fig. 6, *lower-*

right, diSia), demonstrating that the unmasking effect of diSia clustering requires a K_D of at least 5.87×10^{-10} M based on the BLI-based measurement. Using this glycopolymer, a natural ligand could be identified (Yoshimura et al. 2021a).

For multivalency, GD3 expression experiments are interesting (Hashimoto et al. 2019). A colon cancer cell line, DLD-1, which expresses no ligands for the recombinant protein Siglec7-Fc on the cells, showed no Siglec7 binding even after transfection with *ST8Sia1*, responsible enzyme for GD3. After transfection with *ST8Sia1*, GD3 was confirmed to be expressed in DLD-1 cells using an anti-GD3 antibody. However, the addition of purified GD3 from milk to DLD-1 cells resulted in Siglec7 binding. These authors found that Siglec7 recognizes only GD3-containing regular ceramides, but not phytoceramides. The key enzyme was sphingolipid delta(4)-desaturase/C4-monooxygenase (DES2), and the ceramide moiety involved in raft location (clustering in raft domain) and ligand length from the membrane might be important.

5 Natural Ligands for Siglec7

From the viewpoint of natural ligands, three disialogangliosides, namely, disialylgalactosylgloboside (DSGG), $IV^3Neu5AcIII^6Neu5AcLc_4$ ($DSLc_4$), and $IV^4GalNAcIV^3Neu5AcIII^6Neu5AcLc_4$ ($GalNAcDSLc_4$) (Fig. 7), related to lung metastasis were first identified in renal cell carcinoma (RCC) (Ito et al. 2001). Next, the glycan epitope-responsible genes to synthesize the diSia-epitope were considered. The first gene identified was *ST8SIA1*. Transfection approach showed that Siglec7-mediated Sia recognition modulates NK cell killing activity and has important implications in tumor cell escape from NK cell cytotoxicity (Nicoll et al. 2003). The masking of Siglec7 on NK cells was demonstrated using the 2,8-PAA probe because Siglec7 could bind 2,8-PAA only after sialidase treatment. Activation by interleukin (IL)-2, IL-12, and interferon did not favor any binding toward 2,8-PAA, indicating that Siglec7 was constitutively masked by cis-ligands on NK cells. Overexpression of *ST8Sia1* in P815 cells (target cells) led to higher binding toward Siglec7, suggesting that the newly synthesized GD3 is a potential ligand for Siglec7. Inhibition of NK cell cytotoxicity via DSGB4, a ligand for Siglec7, and Siglec7 interaction was also confirmed (Kawasaki et al. 2010). In 2004, $diSiaLe^a$ was considered as a natural ligand for Siglec7 by gene analysis, and synthetic glycan, $diSiaLe^a$, and $diSiaLe^c$ were used for the analysis of Siglec7 binding (Miyazaki et al. 2004). Although the authors did not purify the glycoconjugates, the results from cell-based and in vitro analyses were consistent.

For the bacterial ligand, *Campylobacter jejuni* strains were analyzed (Avril et al. 2006b). Among the Siglec-tested strains, HS:19($GM1^+$, $GT1a^+$) and HS($GD3^+$) showed binding toward Siglec7 in a Sia-dependent manner. HS($GM1^+$, $GD1a^+$) also showed significant binding properties, but the reaction was Sia-independent (Fig. 7, LPS).

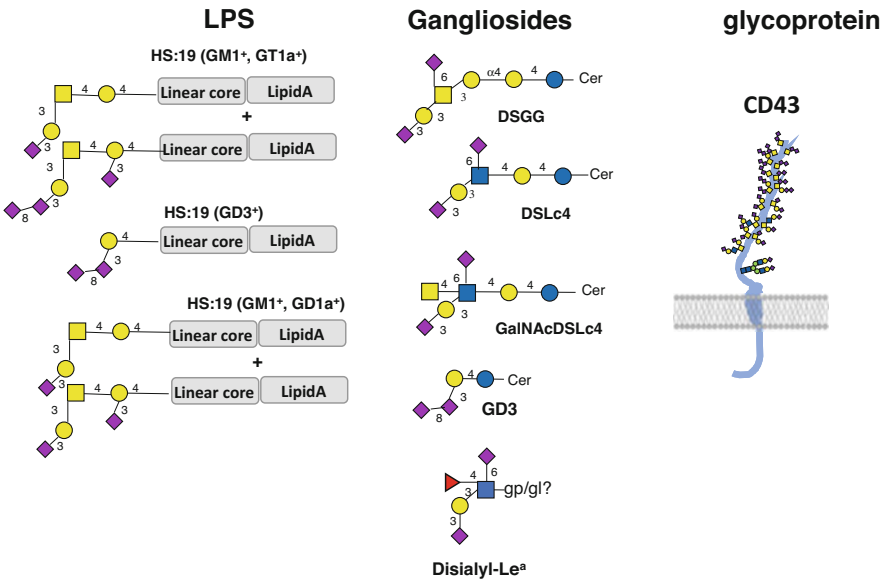

Fig. 7 Natural ligands identified so far. *Campylobacter jejuni* strains have ligands for Siglec7, and HS:19 ($GM1^+$, $GT1a^+$) and HS ($GD3^+$) strains showed binding toward Siglec7 in a Sia-dependent manner. HS ($GM1^+$, $GD1a^+$) also showed significant binding properties but in a Sia-independent manner. Ligands from cancer cells are shown. DSGG, DSLc4, and GalNAcDSLc4 were identified. Using transfection experiments, disialyl Le^a and GD3 were shown to be Siglec7 ligands. From K562 cells, CD43 was identified as a Siglec7 ligand. Ligand on 562 was trapped on a Siglec7 column and specifically eluted using diSia-dex (see Fig. 5). Same results were obtained with the CRISPR system

The natural ligand between NK and K562 cells was analyzed in part. Although NK cells are usually masked by their surface-expressed Sia-containing glycoconjugates, the unmasking system remains unsolved. After unmasking, Siglec7 can bind to tumor cells (K562 cells) because it is a specific inhibitory receptor that checks self and non-self molecules. Therefore, in 2021, proximity labeled molecules on K562 cells were affinity-purified using a Siglec7 column, and specifically bound molecules were eluted with the high-affinity multivalent ligand molecule, diSia-dex (Yamakawa et al. 2020). After mass spectrometry (MS) analysis, the natural ligand of K562 was found to be sialophorin (SPN). At the same time, the possibility of ganglioside ligand expression on K562 cells was analyzed by Siglec7 blotting using TLC immunoblotting; no binding was observed, although the diSia-epitope-containing ganglioside GD3, which was shown to be the Siglec7 ligand using glycan array and enzyme-linked immunosorbent assays (ELISAs), was detected. These data show that the ganglioside on K562 might not be the ligand for Siglec7 and that the trans-natural ligand was SPN. Interestingly, genetically engineered cell-based experiments showed that SPN was a ligand for Siglec7 (Wisnovsky et al. 2021). SPN, also known as leukosialin or CD43, is a type I mucin-type glycoprotein composed of a 19-amino acid signal peptide, an extracellular 255 amino acid region with many O-glycans, and one *N*-glycan near the

transmembrane region (N239). The intracellular 123 amino acid region is related to signal transduction and activation of β-catenin, nuclear factor kappa B, nuclear factor of activated T cells (NFAT), and activator protein-1. The conformational structure of the extracellular domain of SPN shows a rodlike structure, protruding approximately 45 nm from the cell surface. There are approximately 80 *O*-linked glycans in the Ser/Thr-rich region. Based on mutation analysis, it was shown that the Siglec7-binding motif is present at the N terminus of CD43, which is densely glycosylated with disialyl core 1 epitopes and is a favorable location for sensing Siglec7. Further detailed analyses are imperative.

6 Future Perspective

Multivalency is a key feature in understanding natural ligands and drug targeting. Under natural conditions, ligands on glycoproteins and glycolipids are different. In the case of glycoproteins, the natural ligand is localized on the *O*-linked glycan on mucin-type glycoprotein (CD43) (Fig. 8). In the case of glycolipids, especially GD3,

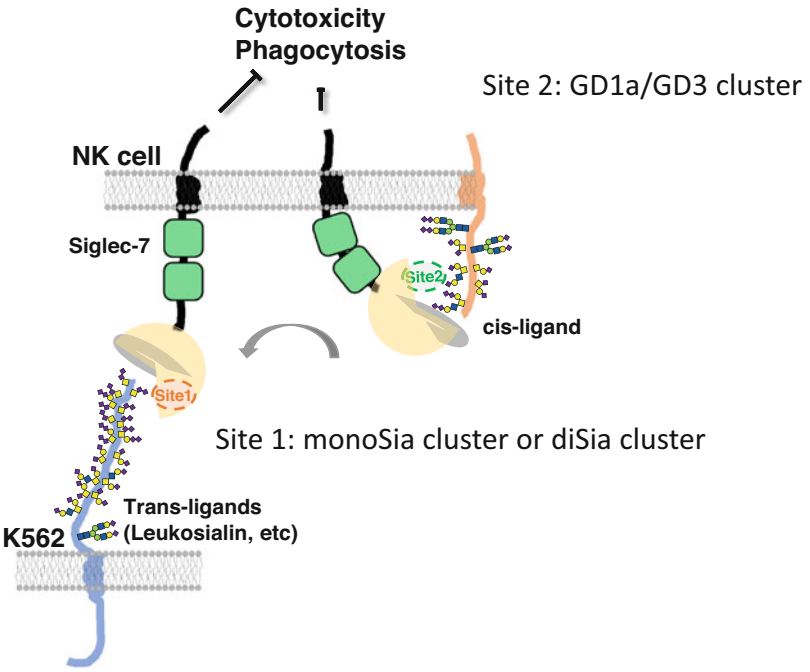

Fig. 8 Hypothesis of the cis/trans ligands of Siglec7. The cells naturally expressing Siglec7, such as NK cells, have masked Siglec7 (masking) because α2,8-PAA showed no binding toward NK cells. Therefore, the cis ligand might be a ganglioside cluster or other glycoproteins on several ligands. NK cells sense cancer cells such as K562 cells when in their vicinity. K562 cells displayed highly dense *O*-lined glycan. NK cells bind to K562 via Siglec7 and mediate immunosuppression. Siglec7 may utilize site 2 and site 1 for cis and trans binding, respectively

the ceramide moiety for localization in the raft and, therefore, the clustering is important. From the viewpoint of synthetic glycopolymers, especially considering binding parameters, clustering ligands such as diSia-dex are the only ligand type that showed the Siglec7-releasing effect from GD3 on the plate. Thus, the affinity of the trans-ligand might be around 10^{10}. Another point is the presence of (at least two) Sia-binding sites on Siglec7 (Fig. 8). Attempts to overcome this affinity have been successful, and in some cases, materials have been used for clinical therapy (Xiao et al. 2016); however, the precise ligand-binding and ligand-releasing mechanisms of Siglecs still remain unclear. A comprehensive consideration of several in silico and in vitro experiments is warranted in the future.

References

Alphey M, Attrill H, Crocker P, van Aalten D (2003) High resolution crystal structures of Siglec-7. Insights into ligand specificity in the Siglec family. J Biol Chem 278:3372–3377

Angata T, Varki A (2000) Siglec-7: a sialic acid-binding lectin of the immunoglobulin superfamily. Glycobiology 10:431–438

Attrill H, Imamura A, Sharma RS, Kiso M, Crocker PR, van Aalten DM (2006a) Siglec-7 undergoes a major conformational change when complexed with the alpha(2,8)-disialylganglioside GT1b. J Biol Chem 281:32774–32783

Attrill H, Takazawa H, Witt S, Kelm S, Isecke R, Brossmer R, Ando T, Ishida H, Kiso M, Crocker PR, van Aalten DM (2006b) The structure of siglec-7 in complex with sialosides: leads for rational structure-based inhibitor design. Biochem J 397:271–278

Avril T, North S, Haslam S, Willison H, Crocker P (2006a) Probing the cis interactions of the inhibitory receptor Siglec-7 with alpha2,8-disialylated ligands on natural killer cells and other leukocytes using glycan-specific antibodies and by analysis of alpha2,8-sialyltransferase gene expression. J Leukoc Biol 80:787–796

Avril T, Wagner ER, Willison HJ, Crocker PR (2006b) Sialic acid-binding immunoglobulin-like lectin 7 mediates selective recognition of sialylated glycans expressed on campylobacter jejuni lipooligosaccharides. Infect Immun 74:4133–4141

Brunetta E, Fogli M, Varchetta S, Bozzo L, Hudspeth KL, Marcenaro E, Moretta A, Mavilio D (2009) The decreased expression of Siglec-7 represents an early marker of dysfunctional natural killer-cell subsets associated with high levels of HIV-1 viremia. Blood 114:3822–3830

Büll C, Heise T, Adema GJ, Boltje TJ (2016) Sialic acid mimetics to target the sialic acid-Siglec axis. Trends Biochem Sci 41:519–531

Chen WC, Kawasaki N, Nycholat CM, Han S, Pilotte J, Crocker PR, Paulson JC (2012a) Antigen delivery to macrophages using liposomal nanoparticles targeting sialoadhesin/CD169. PLoS One 7:e39039

Chen WC, Sigal DS, Saven A, Paulson JC (2012b) Targeting B lymphoma with nanoparticles bearing glycan ligands of CD22. Leuk Lymphoma 53:208–210

Crocker PR, Paulson JC, Varki A (2007) Siglecs and their roles in the immune system. Nat Rev Immunol 7:255–266

Dimasi N, Moretta A, Moretta L, Biassoni R, Mariuzza RA (2004) Structure of the saccharide-binding domain of the human natural killer cell inhibitory receptor p75/AIRM1. Acta Crystallogr D Biol Crystallogr 60:401–403

Hashimoto N, Ito S, Tsuchida A, Bhuiyan RH, Okajima T, Yamamoto A, Furukawa K, Ohmi Y (2019) The ceramide moiety of disialoganglioside (GD3) is essential for GD3 recognition by the sialic acid-binding lectin SIGLEC7 on the cell surface. J Biol Chem 294:10833–10845

Hudak JE, Canham SM, Bertozzi CR (2014) Glycocalyx engineering reveals a Siglec-based mechanism for NK cell immunoevasion. Nat Chem Biol 10:69–75

Ikehara Y, Ikehara SK, Paulson JC (2004) Negative regulation of T cell receptor signaling by Siglec-7 (p70/AIRM) and Siglec-9. J Biol Chem 279:43117–43125

Ito A, Handa K, Withers DA, Satoh M, Hakomori S (2001) Binding specificity of siglec7 to disialogangliosides of renal cell carcinoma: possible role of disialogangliosides in tumor progression. FEBS Lett 504:82–86

Kawasaki Y, Ito A, Withers DA, Taima T, Kakoi N, Saito S, Arai Y (2010) Ganglioside DSGb5, preferred ligand for Siglec-7, inhibits NK cell cytotoxicity against renal cell carcinoma cells. Glycobiology 20:1373–1379

Miyazaki K, Ohmori K, Izawa M, Koike T, Kumamoto K, Furukawa K, Ando T, Kiso M, Yamaji T, Hashimoto Y, Suzuki A, Yoshida A, Takeuchi M, Kannagi R (2004) Loss of disialyl Lewis(a), the ligand for lymphocyte inhibitory receptor sialic acid-binding immunoglobulin-like lectin-7 (Siglec-7) associated with increased sialyl Lewis(a) expression on human colon cancers. Cancer Res 64:4498–4505

Nicoll G, Ni J, Liu D, Klenerman P, Munday J, Dubock S, Mattei MG, Crocker PR (1999) Identification and characterization of a novel Siglec, Siglec-7, expressed by human natural killer cells and monocytes. J Biol Chem 274:34089–34095

Nicoll G, Avril T, Lock K, Furukawa K, Bovin N, Crocker P (2003) Ganglioside GD3 expression on target cells can modulate NK cell cytotoxicity via siglec-7-dependent and -independent mechanisms. Eur J Immunol 33:1642–1648

Ohira S, Yasuda Y, Tomita I, Kitajima K, Takahashi T, Sato C, Tanaka H (2017) Synthesis of end-functionalized glycopolymers containing α(2,8) disialic acids via π-allyl nickel catalyzed coordinating polymerization and their interaction with Siglec-7. Chem Commun (Camb) 53: 553–556

Prescher H, Gütgemann S, Frank M, Kuhfeldt E, Watzl C, Brossmer R (2015) Synthesis and biological evaluation of 9-N-oxamyl sialosides as Siglec-7 ligands. Bioorg Med Chem 23: 5915–5921

Prescher H, Frank M, Gütgemann S, Kuhfeldt E, Schweizer A, Nitschke L, Watzl C, Brossmer R (2017) Design, synthesis, and biological evaluation of Small, high-affinity Siglec-7 ligands: toward novel inhibitors of cancer immune evasion. J Med Chem 60:941–956

Rapoport E, Mikhalyov I, Zhang J, Crocker P, Bovin N (2003) Ganglioside binding pattern of CD33-related siglecs. Bioorg Med Chem Lett 13:675–678

Rillahan CD, Schwartz E, McBride R, Fokin VV, Paulson JC (2012) Click and pick: identification of sialoside analogues for siglec-based cell targeting. Angew Chem Int Ed Engl 51:11014–11018

Rillahan CD, Schwartz E, Rademacher C, McBride R, Rangarajan J, Fokin VV, Paulson JC (2013) On-chip synthesis and screening of a sialoside library yields a high affinity ligand for Siglec-7. ACS Chem Biol 8:1417–1422

Rosenstock P, Horstkorte R, Gnanapragassam VS, Harth J, Kielstein H (2017) Siglec-7 expression is reduced on a natural killer (NK) cell subset of obese humans. Immunol Res 65:1017–1024

Sato C, Kitajima K, Inoue S, Seki T, Troy FA, Inoue Y (1995) Characterization of the antigenic specificity of four different anti-(alpha 2-->8-linked polysialic acid) antibodies using lipid-conjugated oligo/polysialic acids. J Biol Chem 270:18923–18928

Shao JY, Yin WW, Zhang QF, Liu Q, Peng ML, Hu HD, Hu P, Ren H, Zhang DZ (2016) Siglec-7 defines a highly functional natural killer cell subset and inhibits cell-mediated activities. Scand J Immunol 84:182–190

Spence S, Greene MK, Fay F, Hams E, Saunders SP, Hamid U, Fitzgerald M, Beck J, Bains BK, Smyth P, Themistou E, Small DM, Schmid D, O'Kane CM, Fitzgerald DC, Abdelghany SM, Johnston JA, Fallon PG, Burrows JF, McAuley DF, Kissenpfennig A, Scott CJ (2015) Targeting Siglecs with a sialic acid-decorated nanoparticle abrogates inflammation. Sci Transl Med 7: 303ra140

Varchetta S, Brunetta E, Roberto A, Mikulak J, Hudspeth KL, Mondelli MU, Mavilio D (2012) Engagement of Siglec-7 receptor induces a pro-inflammatory response selectively in monocytes. PLoS One 7:e45821

Varchetta S, Mele D, Lombardi A, Oliviero B, Mantovani S, Tinelli C, Spreafico M, Prati D, Ludovisi S, Ferraioli G, Filice C, Aghemo A, Lampertico P, Facchetti F, Bernuzzi F, Invernizzi P, Mondelli MU (2016) Lack of Siglec-7 expression identifies a dysfunctional natural killer cell subset associated with liver inflammation and fibrosis in chronic HCV infection. Gut 65:1998–2006

Wisnovsky S, Möckl L, Malaker SA, Pedram K, Hess GT, Riley NM, Gray MA, Smith BAH, Bassik MC, Moerner WE, Bertozzi CR (2021) Genome-wide CRISPR screens reveal a specific ligand for the glycan-binding immune checkpoint receptor Siglec-7. Proc Natl Acad Sci U S A 118:e2015024118

Xiao H, Woods EC, Vukojicic P, Bertozzi CR (2016) Precision glycocalyx editing as a strategy for cancer immunotherapy. Proc Natl Acad Sci U S A 113:10304–10309

Yamaguchi S, Yoshimura A, Yasuda Y, Mori A, Tanaka H, Takahashi T, Kitajima K, Sato C (2017) Chemical synthesis and evaluation of a Disialic acid-containing dextran polymer as an inhibitor for the interaction between Siglec 7 and its ligand. Chembiochem 18:1194–1203

Yamaji T, Teranishi T, Alphey MS, Crocker PR, Hashimoto Y (2002) A small region of the natural killer cell receptor, Siglec-7, is responsible for its preferred binding to alpha 2,8-disialyl and branched alpha 2,6-sialyl residues. A comparison with Siglec-9. J Biol Chem 277:6324–6332

Yamakawa N, Yasuda Y, Yoshimura A, Goshima A, Crocker PR, Vergoten G, Nishiura Y, Takahashi T, Hanashima S, Matsumoto K, Yamaguchi Y, Tanaka H, Kitajima K, Sato C (2020) Discovery of a new sialic acid binding region that regulates Siglec-7. Sci Rep 10:8647

Yoshimura A, Asahina Y, Chang LY, Angata T, Tanaka H, Kitajima K, Sato C (2021a) Identification and functional characterization of a Siglec-7 counter-receptor on K562 cells. J Biol Chem 296:100477

Yoshimura A, Hatanaka R, Tanaka H, Kitajima K, Sato C (2021b) The conserved arginine residue in all Siglecs is essential for Siglec-7 binding to sialic acid. Biochem Biophys Res Commun 534:1069–1075