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Therapeutic Targeting of Siglecs using Antibody- and Glycan-based Approaches

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Abstract

The sialic acid-binding immunoglobulin-like lectins (Siglecs) are a family of immunomodulatory receptors whose functions are regulated by their glycan ligands. Siglecs are attractive therapeutic targets because of their cell-type specific expression pattern, endocytic properties, high expression on certain lymphomas/leukemias, and ability to modulate receptor signaling. Siglec-targeting approaches with therapeutic potential encompass antibody- and glycan-based strategies. Several antibody-based therapies are in clinical trials and continue to be developed for the treatment of lymphoma/leukemia and autoimmune disease, while the therapeutic potential of glycan-based strategies for cargo-delivery and immunomodulation is a promising new approach. Here, we review these strategies with special emphasis on emerging approaches and disease areas that may benefit from targeting the Siglec family.

Keywords

Siglecs; Sialic acid; Glycans; Antibodies; Synthetic ligands

The Siglec family of immunomodulatory receptors

The Siglec family consists of 15 family members in humans that are expressed on a restricted set of cells in the hematopoietic lineage, with several notable exceptions such as Siglec-4/MAG on oligodendrocytes and Schwann cells and Siglec-6 on placental trophoblasts (Figure 1) [1-3]. Through their outermost N-terminal V-set domain, Siglecs recognize sialic acid-containing glycan ligands on glycoprotein and glycolipids with unique, yet overlapping, specificities. Recognition of their ligands can affect cellular signaling through immunoreceptor tyrosine-based inhibitory motifs (ITIMs) on their cytoplasmic tails. For the majority of the Siglecs, these ITIMs have the capacity of recruiting phosphatases, therefore, these members are referred to as inhibitory-type Siglecs. Exceptions to this are

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Siglec-1 and MAG, which lack such a motif, and the activatory-type Siglecs (Siglecs14-16), which are associated with immunoreceptor tyrosine-based activatory motif (ITAM)-bearing adapter proteins through a positively charged amino acid in their transmembrane region. There are numerous ways in which engagement of Siglecs with their ligands imparts a physiological response, which have been recently reviewed elsewhere [1].

Siglecs as therapeutic targets

The restricted expression pattern of Siglecs makes this family candidate targets for developing therapeutics for the treatment of a wide range of diseases. Nevertheless, it is worth noting that our knowledge of expression patterns of human Siglecs - particularly recently discovered members - is still incomplete and some Siglecs turn up in unexpected places [4-6], necessitating systematic studies. Moreover, the high expression of certain Siglecs on various lymphomas and leukemias has made them obvious targeting candidates [7-9]. However, several additional features of the Siglecs make them particularly well suited for targeting in disease. One important attribute is that most of the Siglecs are rapidly endocytosed upon engagement with antibodies [10-14] or glycan ligands [15-18]. In fact, for certain Siglecs it has been shown that they undergo constitutive endocytosis and recycle back to the cell surface [10,19], although it remains to be determined if this recycling feature is a common feature of the entire Siglec family. Also unresolved is whether the endocytic properties of Siglecs are integral to their natural functions, partly because the endocytosis depends on the sequence motif nested in ITIM and thus mutagenesis will affect both signaling and endocytic properties of Siglecs. Regardless, the endocytic properties of the Siglecs makes them good targets for delivery of cargo to specific cell types. Another characteristic of Siglecs that makes them attractive therapeutic targets is their ability to modulate cellular signaling. Recently developed strategies aim to take advantage of this property to alter cell fate [20]. A growing body of work has shown that these therapeutically relevant properties of Siglecs can be exploited with antibody- and glycan-based targeting strategies. Here we review these strategies by comparing and contrasting the benefits of either strategy, describe diseases that are currently the focus of Siglec targeting, and highlight recent work that has indicated where Siglec targeting may find utility in the future.

Antibody-based targeting of Siglecs

Dating back to the 1980s, the high and selective expression of CD33 and CD22 on certain types of lymphoma and leukemia suggested these Siglecs would be prime candidate targets to treat these cancers. Antibodies against CD22 and CD33 continue to be explored as potential therapeutics against B cell leukemia/lymphoma and acute myeloid leukemia (AML), respectively. In recent years, the antibody-based tools to target Siglecs are becoming more sophisticated (Figure 2), and the range of diseases considered as targets of therapeutic antibodies against Siglecs has expanded (Table 1).

Anti-Siglec antibodies for cell depletion

Antibodies that target Siglecs can deplete the Siglec-expressing cells via recruitment of effector cells of the immune system, delivery of a drug or toxin, or direct induction of an apoptotic signal. Many Siglecs undergo rapid internalization upon ligation by antibody,

which can diminish antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC), and therefore, different approaches can take advantage of the inherent endocytic properties of Siglecs. These include the development of anti-Siglec antibodies conjugated to cytotoxic drugs, as well as the development of antibody-based therapeutics that do not crosslink Siglecs, but rather function by co-engagement of Siglecs with other receptors to enhance the efficiency of effector cell recruitment to the Siglec-expressing target cells.

Antibody-drug and antibody-toxin conjugates

In 2000, Mylotarg (gemtuzumab ozogamicin), an anti-CD33 antibody-calicheamicin conjugate, was approved for the treatment of AML. However, in 2010, Mylotarg was withdrawn from the market because a post-approval clinical trial demonstrated no benefit of adding Mylotarg to the standard chemotherapy for AML, coupled with a greater number of adverse events in the Mylotarg-treated group [9,21]. Nevertheless, retrospective analysis of clinical outcomes has indicated that the efficacy of Mylotarg treatment is influenced by polymorphisms of CD33 (Box 1), which likely decreases its overall effectiveness in total patient populations [22]. Accordingly, renewed efforts are underway to develop next generation antibody-based therapeutics to target CD33 for treatment of AML (Table 1).

The high expression of CD22 on many B cell lymphomas continues to make it an attractive therapeutic target. Although an anti-CD22 antibody has yet to reach the market for the treatment of a B cell leukemia/lymphoma, several compounds are in Phase II and III clinical trials (Table 1). Among the most advanced is inotuzumab ozogamycin [23], which is in Phase III clinical trial for relapsed or refractory B-cell acute lymphoblastic leukemia (B-ALL).

Bispecific antibodies

As chimeric proteins consisting of two antigen-binding modules derived from different antibodies, bispecific antibodies increase the specificity of targeting or enable the crosslinking of target cells and effector cells. Many innovative approaches have enabled the production of bispecific antibodies [24], and recently the first bispecific antibody (blinatumomab) was approved in the U.S. for the treatment of B-ALL. Bispecific antibodies that recognize CD22 and CD19 [25] or CD22 and CD20 [26] on B cells have been developed and, remarkably, show improved efficacy compared to targeting either receptor alone. Bispecific antibodies that co-engage CD33 on AML cells with CD3 on T cells [27,28] or CD16 on natural killer (NK) cells [29] have also shown promising results in pre-clinical and early phase clinical trials (Table 1).

Chimeric antigen receptor (CAR)

CARs are chimeric recombinant membrane proteins consisting of an antibody-derived extracellular domain (e.g., single-chain variable fragment; scFv), followed by a transmembrane domain and intracellular signal transduction domain that activates T cells to enhance the killing of target cells [30]. The production of CAR-T cells requires *ex vivo* culture of effector T cells, ectopic expression of the CAR, and introduction of the modified cells back into the patient. Despite the inherent complexity of this strategy, a Phase I clinical

trial of CD19-targeting CAR-T cells has yielded very promising results [31]. CARs against CD22 [32] and CD33 [33,34] have been developed and tested in early phase clinical trials for the treatment of B-cell lymphoma/leukemia and AML, respectively (Table 1).

Anti-Siglec-8 antibodies for therapeutic depletion of eosinophils

While antibody-mediated recruitment of effector cells or delivery of cytotoxic drug are reliable strategies for depleting individual cell populations, several reports have also indicated that anti-CD22 and anti-CD33 antibodies can directly induce apoptosis in B cell lymphoma and AML cells, respectively [7,21]. It is unclear whether this modest induction of apoptosis contributes to the efficacy that anti-CD22 and anti-CD33 antibodies have achieved. By contrast, a more pronounced apoptotic signal has been shown to occur upon crosslinking of Siglec-8 [35]. Siglec-8 is primarily expressed on eosinophils and mast cells, and induces cell death upon antibody-mediated crosslinking, which can be apoptotic or necrotic depending on the cytokine environment [36-38]. Accordingly, anti-Siglec-8 antibodies have been proposed for treating diseases mediated by eosinophils and/or mast cells, such as bronchial asthma [35]. Interestingly, a therapeutic effect of auto-antibodies against Siglec-8 in intravenous immunoglobulin (IVIG) preparations has been reported [39], rendering support for the potential of such an approach. Antibodies against Siglec-F, a mouse counterpart of human Siglec-8, induces apoptosis and deplete eosinophils in mice [40], thereby alleviating mouse models of eosinophilic diseases such as eosinophilic inflammation of airways [41] and eosinophilic esophagitis [42]. These studies imply the feasibility of anti-Siglec-8 antibody-based therapy for eosinophilic diseases, although it is worth noting that differences between human and mouse Siglecs require consideration (Box 2).

Anti-Siglec antibodies for functional modulation—While the depletion of the cells that express particular Siglec can be an effective means of treating certain diseases, target cell killing is not always the preferred treatment strategy. For example, depletion of this cell population could lead to another disease. An alternative approach is to take advantage of the natural regulatory functions of Siglecs to modify the behavior of the cells that express Siglecs. Antibodies that modify the function of Siglecs are emerging as an efficacious means of treating a range of diseases.

Anti-CD22 antibodies for the treatment of systemic lupus erythematosus (SLE)

Epratuzumab is a humanized unconjugated monoclonal antibody against CD22 in Phase III clinical trials for the treatment of SLE, which is a B cell-driven autoimmune disease [43,44]. Although epratuzumab can deplete CD22+ B cells to some extent, other modalities are thought to be at work [45]. Modulation of B cell signaling by inducing trogocytosis of cell-surface proteins [46], modulation of adhesion molecule expression [47], and suppression of B cell receptor (BCR) signaling [48], have been proposed as contributing modes of action. A more complete understanding for the efficacy that epratuzumab has shown in Phase II clinical trials may provide additional insights on how to target CD22 for the treatment of autoimmune disease.

Anti-MAG/Siglec-4 antibodies for treatment of neuronal degeneration

Myelin-associated glycoprotein (MAG)/Siglec-4 is expressed on myelinating cells (oligodendrocytes and Schwann cells) and negatively regulates neuronal regeneration, and thus blockade of its inhibitory function may be beneficial when neuronal regeneration is necessary, such as after a stroke [49]. One study has shown that systemic administration of an antibody against MAG can facilitate functional recovery in an animal model of stroke [50]. A humanized antibody against MAG (GSK249320) has been developed and tested in Phase I clinical trials [51,52]. Through undisclosed modifications to the Fc portion of the antibody, ADCC and CDC have been averted to prevent the development of a disease similar to anti-MAG peripheral neuropathy [53].

Anti-Siglec-15 antibodies for treatment of osteoporosis

Siglec-15 is highly expressed on osteoclasts and involved in bone homeostasis [54-57]. As osteoclasts facilitate osteoporosis and Siglec-15 is critical for proper development and function of osteoclasts, anti-Siglec-15 antibodies have therapeutic potential in treating osteoporosis [58]. Indeed, an antibody against Siglec-15 was developed and tested in a mouse model of osteoporosis, and has shown promising results [11]. It is worth noting that this effect is dependent on antibody-mediated crosslinking, internalization, and lysosomal degradation of Siglec-15.

Auto-/allo-antibodies against Siglec: possible leads to novel therapeutics?

Auto-antibodies against Siglec-8 or Siglec-9 (inhibitory-type Siglecs) may be partly responsible for the anti-inflammatory effect of intravenous immunoglobulin (IVIG) therapy [39,59], while the presence of allo-antibody against Siglec-14 (activatory-type Siglec) is associated with transfusion-related adverse events [60]. These findings imply that crosslinking of inhibitory-type Siglecs may be useful for the treatment of autoimmune or inflammatory diseases, while blocking antibodies (Fab fragment or scFv) against activating-type Siglecs may also be worth exploring.

Perspectives for anti-Siglec antibody-based therapies—Antibodies remain a reliable tool to deliver cargo to a specific cell population that expresses its antigen, and sophisticated technologies based on the antibody scaffold continue to be developed. Due to the restricted expression of Siglecs in certain immune cell types, cell depleting anti-Siglec antibodies also continue to be of value for interrogating the natural function of these cell types in mice, which in turn points to potential therapeutic uses. For example, depletion of eosinophils in mice with an antibody targeting Siglec-F (murine counterpart of human Siglec-8; see Box 2) has become a standard procedure, which has helped to shed light on novel roles for eosinophils and the potential therapeutic benefit of controlling their numbers [61]. Based on recent developments in the Siglec field of research, novel antibody-based therapies can also be envisioned that target CD33 for de-repressing microglial clearance of amyloid plaques in Alzheimer's disease (Box 1) [62,63] or Siglec-7 and Siglec-9 to enhance cancer immunosurveillance by NK cells [64,65] (see Outstanding Questions Box).

Targeting Siglecs using Glycan Ligands

The auxiliary function of antibody (e.g., binding to complement and Fc receptors) can cause serious side effects, and the development of anti-drug antibodies against therapeutic antibodies can preclude their long-term use. Using glycan ligands to target the Siglec family represents an alternative approach to antibody-based strategies, and offers several advantages such as lack of auxiliary function, the general lower immunogenicity of glycans compared to proteins [66], and the ability to dissociate under mild physiological conditions. Compared to antibody-based approaches, applications that target Siglecs using glycan ligands are still in their early stages, yet much progress has been made over the past five years. In this section, we outline strategies used for ligand discovery, highlight the current state of available high affinity Siglec ligands, and review proof-of-concept therapeutic strategies where these glycan ligands have been used.

Development of synthetic glycan ligands of Siglecs

General and historical aspects of ligand design—Ligand-based targeting probes use synthetic sialic acid-containing glycans to target the binding site of Siglecs. Importantly, these ligands must be sufficiently potent to overcome masking of the Siglec binding site by endogenous *cis* ligands on the target cell. Multivalent probes displaying high affinity glycan ligands are clearly capable of outcompeting *cis* ligands [67]. Each Siglec exhibits a distinct binding preference for the type of sialic acid, the glycosidic linkage, and the underlying glycan structure [1]. Ligand design strategies incorporate these structural preferences; however, in general Siglecs bind their natural sialoside ligands with low affinity. Therefore, the discovery of selective high affinity Siglec ligands has relied on the development of synthetic sialoside analogs (Figure 3).

In earlier work, the crystal structure of Siglec-1 complexed with α 2-3 sialyllactose suggested that synthetic sialosides modified at C-9 of *N*-acetyl neuraminic acid (Neu5Ac) could enhance affinity [68]. Indeed, appending a hydrophobic biphenyl substituent at the 9-position, to create methyl α -9-*N*-(biphenyl-4-carbonyl)-amino-9-deoxy-Neu5Ac (BPC-Neu5Ac), resulted in a 13-fold increase in potency for Siglec-1 compared to methyl-Neu5Ac. The structure of Siglec-1 complexed with BPC-Neu5Ac revealed that the biphenyl substituent is accommodated in a hydrophobic pocket adjacent to the sialic acid binding site and suggested a strategy for further ligand design [69].

Different ligand design strategies have emerged with respect to the choice of underlying structure at the C-2 position of sialic acid. For instance, development of a first generation high affinity CD22 ligand combined the preferred specificity for the glycan sequence Sia α 2-6Gal β 1-4GlcNAc with C-9 modification [67]. The chemoenzymatically synthesized, 9-BPC-Neu5Ac α 2-6Gal β 1-4GlcNAc (BPC-Neu5Ac-LacNAc) demonstrated a 16-fold increase in potency against CD22 compared to methyl BPC-Neu5Ac. Alternatively, substitution at C-2 of sialic acid with hydrophobic groups has simplified ligand design while providing favorable affinity gains. For example, compared to methyl-Neu5Ac α , benzyl-Neu5Ac α was found to be 8.1 and 9.8-fold more potent against Siglec-1 and Siglec-4, respectively [70,71].

In recent years, various approaches have been employed to develop high affinity Siglec ligands that expand on this strategy by introducing novel substituents at C-9 of sialic acid as well as exploring modifications at C-5, C-4 and C-2 (Figure 3).

Structure-guided ligand design—Crystal structures for the ligand-binding domain of Siglec-1, -5, and -7 have been solved [69,72,73]. The structure of Siglec-1 complexed with methyl BPC-Neu5Ac was recently used to guide the design of a potent Siglec-1 ligand [74]. An *in silico* screen was performed on a commercial library of ~8400 carboxylic acids to identify novel 9-*N*-acyl substituents that could favorably bind to the Siglec-1 hydrophobic pocket occupied by the BPC substituent. As an alternative to solution based screening methods, which requires extensive synthetic effort, this approach has the advantage of rapidly screening large compound libraries to identify lead structures. A small panel of carboxylic acids were identified and the corresponding 9-*N*-acyl Neu5Ac α 2-3Gal β 1-4GlcNAc structures were chemoenzymatically synthesized for solution based assessment of affinity and selectivity. This screen identified a sialoside analog, designated 9-TCC-Neu5Ac α 2-3Gal β 1-4GlcNAc (TCC-Neu5Ac, **1**, Figure 4), which binds selectively to Siglec-1 with an IC_{50} value of 0.38 μ M. As described below, this TCC-Neu5Ac can be used for targeted delivery of liposomal nanoparticles to Siglec-1+ macrophages.

In the absence of a crystal structure, homology models have been constructed for several Siglecs to interpret binding affinities at the molecular level. A homology model of the ligand-binding domain for Siglec-4 was generated based on the three-dimensional structure of Siglec-1 [75]. Docking studies with a potent Siglec-4 inhibitor (**2**, Figure 4, $K_D = 0.5 \mu$ M) suggested that both hydrophilic and hydrophobic interactions were important for binding [76]. Independently, a second homology model of the Siglec-4 ligand binding domain was constructed based on the available crystal structures for Siglec-1, -5 and -7 [77]. Likewise, this model was used to rationalize the binding of a potent Siglec-4 inhibitor (**3**, Figure 4, $K_{D,app} = 0.015 \mu$ M). Moreover, evaluation of a CD22 homology model led to the hypothesis that substituents at C-4 of Neu5Ac may provide additional favorable interactions [78]. From a small library of synthetic C-4 modified sialic acid analogs, inhibition analysis identified an amide substituent that provided 20-fold increased affinity for CD22 compared to methyl BPC-Neu5Ac (**4**, Figure 4, $IC_{50} = 0.15 \mu$ M).

Structure-activity relationship (SAR) guided ligand design—Ligand design has also been facilitated by SAR analysis of sialic acid analog libraries [71,75-81]. Synthetic libraries have been evaluated to identify potential Siglec ligands using, for example, inhibition assays, surface plasmon resonance, and isothermal titration calorimetry. While this approach has led to the development of potent Siglec inhibitors (for example, **2-8**, Figure 4), the disadvantages of this method are the relatively small compound libraries that are produced, and the laborious screening methods to probe the libraries. Recently, a high-throughput strategy was developed for the facile synthesis and rapid screening of sialic acid analog libraries [82]. A glycan library (216 analogs) of Sia α 2-3- and Sia α 2-6-lactose modified at C-9 or C-5 of sialic acid was synthesized via high-throughput copper-I catalyzed azide-alkyne cycloaddition (CuAAC). For high-throughput screening, the resulting library

was printed as a microarray on glass slides and probed simultaneously with recombinant Siglecs to identify high affinity ligands. Lead compounds selective for several Siglecs were identified, including a high affinity ligand for Siglec-9 (**9**, Figure 4), and Siglec-10 (**10**, Figure 4). Subsequently, this approach was expanded by an on-chip synthesis and screening strategy that yielded a high affinity ligand for Siglec-7 (**11**, Figure 4) [83].

Development of high affinity ligands has also been achieved by taking advantage of the additive binding effects from incorporating multiple sialic acid substituents. For instance, a potent human CD22 ligand (**7**, Figure 4, $K_D = 60$ nM) modified at C-9, C-5 and C-2 of sialic acid was developed by systematically identifying and combining favorable substituents for each position [80]. A selective ligand for human CD33 (**12**, Figure 4) and a more potent and selective second-generation ligand for human CD22 (**13**, Figure 4) have also benefited by taking advantage of di-substituted sialic acid [84]. It is also worth noting that a selective ligand for Siglec-G (murine counterpart of Siglec-10, see Box 2) was developed through combining C-9 modification with the preferred glycan sequence Neu5Gc α 2-3Gal β 1-4GlcNAc (**14**, Figure 4) [85]. Similarly, murine CD22 on B cells can be targeted using a ligand combining C-9 modification with the glycan sequence Neu5Gc α 2-3Gal β 1-4GlcNAc (**15**, Figure 4) [86].

Delivering cargo with glycan ligands

Drugs and toxins to cancer cells—The high expression of CD22 on numerous types of B cell lymphomas, the efficient endocytic properties of CD22, and the availability of high affinity and selective ligands of CD22 have made it an ideal target for testing the ability of glycan ligands to deliver chemotherapeutic drugs. Displaying high affinity CD22 ligands on the surface of liposomal nanoparticles results in rapid endocytosis of the nanoparticles via CD22 [15]. Once in endosomes, CD22-glycan ligand interactions are weakened by pH acidification, allowing nanoparticles bearing the CD22 ligands to be dropped off while CD22 returns to the cell surface to pick up more cargo (Figure 5) [19]. This is in contrast to CD22-antibody interactions that remain intact during internalization, resulting in the recycling of the CD22-antibody complex to the cell surface [10,19]. Hence, glycan ligands of CD22 have the remarkable advantage over antibodies in that they allow CD22 to function in an analogous fashion as a pump for the uptake of cargo into the cell. Accordingly, high affinity CD22 ligands appended to liposomes encapsulated with doxorubicin has been shown to be a potent method for killing human B cell lymphoma cells both *in vitro* and in mice [15]. Furthermore, a soluble dimeric high affinity CD22 ligand conjugated to a toxin was shown to be efficient in killing B-ALL cells *in vitro* (Figure 5) [87]. Given the recent development of more potent and selective ligands for other members of the Siglecs, such as CD33 [84], it will be interesting to see if these delivery principles can also be used to target other types of lymphomas and leukemias.

Antigens to antigen-presenting cells (APCs)—Professional APCs, which consist primarily of macrophages, dendritic cells, and B cells, process and present antigen to T cells to prime immune responses. Given that APCs generally take up antigen in a non-specific manner, enhanced delivery of antigen to APCs is considered a method for increasing the efficiency of vaccination strategies [88]. CD169+/Siglec-1+ macrophages are a

physiologically important APC spatially positioned in secondary lymphoid organs for optimal priming of immune responses [89]. Motivated by the fact that Siglec-1 is an endocytic receptor, high affinity and selective Siglec-1 ligands were developed to target cargo to these CD169+ macrophages. Liposomal nanoparticles displaying Siglec-1 ligands were shown to be rapidly endocytosed by Siglec-1-expressing macrophages [16,74]. Using this targeting strategy, both protein and lipid antigens can be delivered to CD169+ macrophages, to enhance activation of CD4+ helper T cells and natural killer T (NKT) cells, respectively (Figure 5) [16,90]. Using a similar liposomal-based targeting strategy, mycobacterial lipid antigens can also be efficiently delivered to human dendritic cells via Siglec-7 to activate CD1b-restricted T cells [17]. Given the diversity of Siglecs expressed on dendritic cells and macrophages, and the efficient endocytic properties of the Siglec family as a whole [1], Siglec ligand-mediated delivery of cargo to APCs is an approach with broad potential.

Modulating cellular signaling

The inhibitory-type Siglecs contain ITIM motifs that are capable of recruiting phosphatases to inhibit immune cell activation [1]. Therefore, recruiting inhibitory-type Siglecs within the appropriate context has the potential to dampen immunity, which could be useful in diseases where unwanted immune cell activation plays a critical role in the disease. Since the immunomodulatory effects imparted by the inhibitory Siglecs are largely driven by spatial proximity to an activatory receptor [1], an understanding of what activatory receptor(s) can be inhibited by a given Siglec is essential. One well-studied example is the ability of CD22 and Siglec-10 (or its mouse counterpart, Siglec-G; Box 2) to inhibit the B cell receptor [91]. Recently, a series of biochemical approaches have demonstrated that high affinity CD22 ligands presented on the same surface as an antigen, can be used to recruit CD22 to the BCR to not only inhibit B cell activation, but also induce antigen-specific B cell tolerance (Figure 5). Initial work in this area was with polymers that display a hapten (non-protein T-independent antigen) and a CD22 ligand, where it was shown that B cell activation is remarkably inhibited by presence of CD22 ligands [86,92]. When the polymer displaying both hapten and high affinity ligand for mouse CD22 was administered to mice, it was remarkable that mice failed to respond to a subsequent challenge with a polymer displaying only the antigen [86].

Subsequently, liposomal nanoparticles bearing a T-dependent (protein) antigen and high affinity CD22 ligands were developed to test whether immunological tolerance can also be induced toward this type of antigen (Figure 5) [93]. Even when a strong T-dependent antigen was used, engaging CD22 with the BCR could prevent an antibody response to that antigen in a subsequent challenge. Tolerance was shown to be the result of apoptosis of B cells recognizing the antigen, which stemmed from inhibition of basal BCR signaling that is essential in B cells. The concept of inducing immunological tolerance by recruiting CD22 to the immunological synapse of cells recognizing the antigen was shown to be potentially useful preventing unwanted antibody responses to biotherapeutics. For example, in hemophilia A approximately 25-30% of patients treated with the blood-clotting factor they are missing (Factor VIII; FVIII) develop antibodies to FVIII that greatly diminishes its effectiveness. In a mouse model of hemophilia A, inducing immunological tolerance to

FVIII by displaying the protein on a liposome along with high affinity CD22 ligands resulted in a failure to generate anti-FVIII antibodies after a subsequent challenge with the antigen and allowed for successful treatment with FVIII to prevent bleeding. Co-engaging Siglec-G with the BCR by creating liposomes that co-display antigen and high affinity ligands specific for Siglec-G were also shown to induce immunological tolerance [85]. Therefore, two Sigslecs on B cells can be independently targeted with Siglec-engaging tolerance-inducing antigenic liposomes (STALs), to suppress antibody responses and induce immunological tolerance [20].

Perspectives for glycan-mediated targeting of Sigslecs

To date, high affinity and selective ligands have been developed for approximately half the Sigslecs in humans. The discovery of high affinity ligands for Siglec-8 may enable eosinophil and mast cell depletion [35,94] and high affinity ligands for Siglec-15 may be useful in blocking the function of Siglec-15 on osteoclasts for the treatment of osteoporosis [58]. As semisynthetic glycan ligands that target the Selectin family are proving to be clinically tractable [95], development of Siglec ligands with even greater potency could enable natural Siglec-ligand interactions to be disrupted. Accordingly, efforts to design second and third generation ligands with increased potency are needed [77,78,80,84]. In doing so, opportunities may emerge for (see Outstanding Questions Box): disrupting, alleviating CD33-mediated inhibition of amyloid plaque uptake by microglia for the treatment of Alzheimer's disease (Box 1) [62], blocking B cell trafficking to the gut [96], inhibiting MAG activity to enhance neuronal regeneration [97], disrupting CD52-Siglec-10 interactions for the treatment of autoimmune disease [98], preventing Siglec-1 interactions with HIV [99], and dampening toll-like receptor signaling in autoimmunity [100]. While hope for these glycan-based therapeutics runs high, thorough evaluation of the antigenicity of these synthetic compounds are warranted in applications where a therapeutic regimen involves repeated administration of the glycan-containing therapeutic, as neutralizing antibodies against them would have the potential to limit their efficacy.

Concluding Remarks

The cell-type specific expression and endocytic properties of Sigslecs make them prime targets for therapeutic intervention. Antibodies targeting individual members of the Siglec family continue to emerge as potential therapeutics in the areas of cancer and autoimmunity; however, new disease areas have also emerged, such as the potential treatment of osteoporosis and neurodegeneration, through targeting of Siglec-15 and MAG, respectively (see Outstanding Questions Box). The number of clinical trials targeting CD22 and CD33 are also increasing, in particular, due to innovative antibody-based strategies such as bispecific antibodies and CAR-T cell therapy. Engineered multi-specific antibodies that target Sigslecs and other cell-surface receptors expressed on the same cell surface may be useful for functional modulation of immune cells, and clinical utility of such reagent can be envisioned. However, the therapeutic potential of high affinity and selective glycan ligands of the Sigslecs is emerging due in large part from pioneering efforts to develop these ligands. In certain applications, glycan ligands have an advantage over antibodies, such as their ability to dissociate from their target once endocytosed. Another application where glycan

ligands could make significant impact is in recruiting Siglecs to the appropriate receptor to modulate cellular signaling and cell fate, as has been recently demonstrated with STALs to induce antigen-specific B cell tolerance. As knowledge of the physiological roles for Siglecs continues to expand, so too will the opportunities for modulating disease through targeting the Siglecs.

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Glossary Box

Antibody-dependent cellular cytotoxicity (ADCC)	A component of the adaptive immune response and an important effector function of antibodies. Antibodies coating the surface of a target cell recruit effector cells (ex. NK cells) that induces target cell lysis
Complement-dependent cytotoxicity (CDC)	Part of the adaptive immune response involving complement activation. Antibodies coating the surface of a target cell recruit C1q, leading to activation of the classical pathway for complement activation and formation of a membrane attack complex that induces target cell lysis
Immunoreceptor tyrosine-based inhibitory motif (ITIM)	Tyrosine residues on the cytoplasmic tail of receptors are phosphorylated under the appropriate physiological circumstances. Phosphorylation creates a ligand for SH2 domain-containing proteins, thereby, recruiting then to modulate immune cell activation
N-acetylneuraminic acid (Neu5Ac)	A 9-carbon keto sugar, which is the most prominent sialic found in mammals. Typically terminates glycans through α 2-6, α 2-3, or α 2,8 linkages with the underlying carbohydrate residue. Installed on glycoproteins and glycolipids through the actions of the sialyltransferase family of glycosyltransferases
Sialic acid-binding immunoglobulin-type lectin (Siglec)	A family of 15 (in humans) cell surface receptors expressed predominantly in the immune system that typically recognizes sialic acid-containing glycoproteins and glycolipids as their ligands. Modulation of immune cell activation is mediated through either ITIMs in their cytoplasmic domain, or through positively-charged residues in their transmembrane domain that mediate pairing with activatory co-receptors. Several Siglecs do not have either an ITIM or a positively charged transmembrane, but are still capable of modulating cellular activation
Siglec-engaging tolerance-inducing antigenic liposomes (STALs)	Liposomes that co-display an antigen and high affinity Siglec ligand. Co-presentation recruits CD22 to the BCR on the surface of B cells, resulting in an apoptotic signal that gives rise to antigen-specific B cell tolerance

Box 1**Clinically relevant polymorphisms of the Siglecs**

Genetic polymorphisms of CD33 are not only relevant to AML treatment with antibody, but also in the development, and possibly treatment, of Alzheimer's disease. Genome-wide association studies have revealed an association between the *CD33* gene and late-onset Alzheimer's disease (LOAD) [101,102]. A likely "causative" variation (rs12459419 T allele) in the *CD33* gene associated with the reduced risk of LOAD was identified, which is a single-nucleotide polymorphism (SNP) near an exon/intron border that increases the proportion of CD33 protein lacking the N-terminal Ig-like domain (Ig1) [103,104]. CD33 is expressed on brain microglia and inhibits the endocytic clearance of insoluble amyloid beta, which is a putative culprit of LOAD development, while the CD33 variant lacking Ig1 is less inhibitory, allowing more efficient clearance [62]. The allele is associated with apparently reduced CD33 expression level, because most anti-CD33 antibodies recognize Ig1 [105]. Interestingly, the same allele is associated with favorable outcomes in the pediatric AML patients treated with Mylotarg [22]. The CD33 allele carried by AML and LOAD patients will surely need to be considered in the development of future therapeutics.

Correlations between genetic polymorphisms of other Siglec genes and diseases have been reported, such as *SIGLEC8* and bronchial asthma [106], *SIGLEC9* and lung cancer [107], *SIGLEC14* and exacerbation of chronic obstructive pulmonary disease [108], and *SIGLEC14* and premature labor [5]. Null polymorphisms of *SIGLEC12* and *SIGLEC16* genes are also known [6,109], which may influence clinical parameters (e.g., susceptibility or prognosis) of some diseases and of potential interest. Genetic polymorphisms may also manifest themselves in the different expression patterns of Siglecs among different individuals, as shown for Siglec-5 [108], demonstrating clear need for further studies of expression patterns of human Siglecs. These associations not only validate the therapeutic approaches targeting these Siglecs, but also caution that genetic variations in evaluating efficacy of antibody-based therapies must be considered.

Box 2**Pre-clinical targeting of Siglecs in mice: differences between human and mouse Siglecs**

As a rapidly evolving family of receptors, numerous differences exist between human and mouse Siglecs. Some of these differences stem from gene deletions and duplications that, overall, have resulted in humans having 15 Siglecs, while mice have only 9 Siglecs. Pairs of human/mouse Siglecs that are established counterparts are: hSiglec-1/mSiglec-1, hCD22/mCD22, hMAG/mMAG, hSiglec-10/mSiglec-G, and hSiglec-15/mSiglec-15 (true orthologs); hCD33/mCD33 and hSiglec-9/mSiglec-E (putative orthologs); and hSiglec-8/mSiglec-F (not orthologs, but functionally convergent counterparts). Even within these conserved members, there are notable differences in ligand specificity, which is partly due to differences in sialic acid between the two species: humans have exclusively Neu5Ac, whereas mice have both Neu5Ac and Neu5Gc [110].

Considerations of ligand specificity have obvious implications in the development of high affinity targeting ligands, which relates to pre-clinical studies in mice. Different ligands may need to be developed and used for pre-clinical studies in mice, than would be used in humans. An example is targeting of CD22, which has subtle differences in ligand preference, such that different high affinity ligands are required to target human [15] and mouse [86] CD22. More straightforward would be to use mice that express the human Siglec of interest. Indeed, two studies have reported the development of mouse models of human Siglec expression. Human CD22 knock-in mice were made, although its expression on mouse B cells is significantly lower than on human B cells [111]. More recently, transgenic mice expressing Siglec-9 on a background lacking its functional counterpart (Siglec-E) were produced [107]. It is noteworthy that the ligand specificity of the relevant Siglec should be considered in relation to the types of sialic acid ligands expressed on the cell type of interest is an important consideration, since the levels of *cis* ligands strongly influences the ability of Siglecs to recognize ligands in *trans*. Overall, further development of mice expressing human Siglecs appears warranted for pre-clinical investigation of targeting the Siglec family for therapeutic benefit.

Outstanding Questions Box

- Can antibodies to Siglec-7 and/or Siglec-9 be useful therapeutically in enhancing killing of cancer cells by NK cells?
- Can antagonists of CD33 be useful in enhancing amyloid plaque uptake by microglial cells?
- Can high affinity and selective ligands towards Siglec-8 and Siglec-15 be developed for potential therapeutic use in asthma and osteoporosis?
- In a similar fashion as shown for the B cell Siglecs, can high affinity Siglec ligands be used to modulate the immune response for potential therapeutic intervention?
- Can Siglec ligands with sufficiently high affinity be developed to block endogenous Siglec-ligand interactions?
- Can selective sialyltransferase inhibitors or inhibitors of glycan modifying enzymes (ex. sulfotransferases) be developed to modulate Siglec-ligand interactions?
- What is the best strategy to integrate the information of Siglec polymorphisms into antibody-based therapies? Patient stratification (or otherwise)?

Trends Box

- Siglecs are attractive therapeutic targets due their restricted expression pattern.
- The endocytic and immunomodulatory properties of Siglecs can be exploited.
- Numerous antibody-based therapies targeting Siglecs are in clinical development.
- Glycan ligands of Siglecs are effective for cargo delivery and functional modulation.

Figure 1.

The family of human Siglecs. Differences between family members include the number of extracellular Ig domains, the number of intracellular ITIM motifs, the presence of a positively charged intramembrane residue (Siglecs 14-16), and loss of sialic acid recognition (Siglec-12). Inhibitory-type and activatory-type Siglecs are noted. Expression patterns for each Siglec in normal individuals are indicated below: Mac, macrophage; B, B cell; Mon, monocyte; MyP, myeloid precursor; OligD, oligodendrocyte; Neu, neutrophil; NK, natural killer cell; Eos, eosinophil; Bas, basophil; Mast, mast cell; DC, dendritic cell; Epi, epithelial cell; Osclast, osteoclast.

(a) Antibody and antibody conjugate **(b) Bi-specific antibody** **(c) Chimeric antigen receptor (CAR)** **Figure 2.**

Antibody-based approaches under development for therapeutic targeting of the Siglecs. (a) Unconjugated antibodies, antibody-drug conjugates, and antibody-protein toxin conjugates (also known as immunotoxin) are traditional antibody-based targeting approaches, but the clinical properties of these therapeutic molecules continue to be improved, such as the stability/cleavability of the linker between antibody and drug or toxin as well as the antigenicity of the protein toxin. Not represented, but also under development, are radionuclide-conjugated antibodies. (b) Advancements in the design and production of bispecific antibodies have yielded a few cases of Siglec-targeting bispecific antibodies that are in early phase clinical development. (c) A chimeric antigen receptor (CAR) consists of single-chain variable fragment (scFv) of an antibody, followed by a linker peptide and a combination of transmembrane/intracellular signaling domains of T cell receptor (CD3ζ) and co-stimulatory receptor (e.g., CD28 or CD137/4-1BB). Primary T cells from the patient are virally transduced with the construct to express CAR, propagated, and returned to the patient's body. CAR-positive T cells are stimulated upon encounter with the antigen-expressing target cells *in vivo*, leading to their expansion and the cytolytic elimination of the target cell population. A few cases of early phase clinical trials of Siglec-targeting CAR have been reported.

Figure 3.

General structure of sialic acid analogs used as high affinity Siglec ligands. Different approaches have been employed to develop high affinity Siglec ligands by introducing various substituents at C-9, C-5, and C-4 of sialic acid. Varied substituents at C-2 of sialic acid have also been used including aromatic aglycones (**A**) and glycans where the modified sialic acid is linked α 2-6 (**B**) or α 2-3 (**C**) to galactose (Gal) of lactose or LacNAc.

Figure 4.

Overview of sialoside analog based high affinity Siglec ligands. Numbering based on discussion of compounds in text. Ph, phenyl. Lactose, Gal β 1-4Glc. LacNAc, Gal β 1-4GlcNAc.

Figure 5.

Glycan ligand-based approaches with therapeutic potential. The therapeutic potential of high affinity Siglec ligands have been shown in the area of delivering drugs or toxins to cancer cells (*upper left*), delivering antigens to APCs (*upper right*), and inducing antigen-specific B cell tolerance (*lower*). For delivery of chemotherapeutic agents to cancer cells, high affinity Siglec ligands have been attached to liposomes encapsulated with drug or directly conjugated to a protein toxin. As has been demonstrated for CD22, following endocytosis into endosomes Siglec-ligand interactions dissociate, allowing the cargo to be dropped off and the Siglec to recycle to the cell surface for another round of delivery. For delivery of antigens to APCs, protein and lipid antigens have been delivered to APCs (macrophages and dendritic cells) via incorporation into Siglec-targeted liposomes. Once inside the cell, antigens are loaded onto the appropriate complex (MHC I, CD1d, or CD1b) and are presented for activation of T cells, NKT cells, or CD1b-restricted T cells, respectively. For

induction of antigen-specific B cell tolerance, Siglec-engaging tolerance-inducing antigenic liposomes (STALs) present both antigen and high affinity CD22 or Siglec-G ligand, which co-engage the Siglecs with the BCR to deliver a pro-apoptotic effect to B cells that recognize the antigen.

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Table 1

Anti-Siglec antibodies and derivatives in clinical development

Target	Name	Type	Disease	Stage	ClinicalTrials.gov Identifier
CD22/Siglec-2	Epratuzumab (clone: LL2)	Unconjugated	SLE	Phase 3	NCT01262365 NCT01408576 NCT01261793
			ALL	Phase 2	NCT00945815
	Inotuzumab ozogamycin (clone: GS/44)	Drug conjugate	ALL	Phase 3	NCT01564784
	Pinatuzumab vedotin (clone: 10F4)	Drug conjugate	NHL	Phase 2	NCT01691898
	Moxetumomab pasudotox (clone: RFB4)	Fv-toxin fusion	ALL	Phase 2	NCT02338050
	BL22 (clone: RFB4)	Fv-toxin fusion	HCL	Phase 2	NCT00924040 (terminated)
	RFB4-dgA (clone: RFB4)	Toxin conjugate	ALL	Phase 1	NCT01408160
	DT2219ARL (clone: RFB4)	Bispecific scFv-toxin fusion (CD22/CD19)	B-cell leukemia/lymphoma	Phase 1/2	NCT02370160
	22*--(20)-(20) (clone: LL2)	Bispecific hexavalent (CD22/CD20)	NHL, SLE	Pre-clinical	N/A
	CD22-CAR (clone: m971)	CAR-T	FL, ALL, NHL, LCL	Phase 1	NCT02315612
CD33/Siglec-3	Gemtuzumab ozogamycin (clone: P67.6)	Drug conjugate	AML	Approved but withdrawn	NCT00085709
	SGN-CD33A (clone: M195)	Drug conjugate	AML	Phase 1 Phase 1	NCT01902329 NCT02326584
	HUM195/rGEL (clone: M195)	Toxin conjugate	AML	Phase 1	NCT00038051
	AVE9633 (clone: My9-6)	Drug conjugate	AML	Phase 1	NCT00543972
	AMG-330 (clone: N/A)	Bispecific (CD33/CD3)	AML	Pre-clinical	N/A
	CAR-T-33 (clone: N/A)	CAR-T	AML	Phase 1/2	NCT01864902
MAG/Siglec-4	GSK249320 (clone: N/A)	Ab (Fc-engineered)	Stroke	Phase 2	NCT01808261 (terminated)
Siglec-15	AB-25E9	Unconjugated	Osteoporosis	Pre-clinical	N/A

“Clone” stands for the original clone ID of anti-Siglec antibody utilized for the drug development. Ab: antibody; ALL: acute lymphoblastic leukemia; AML: acute myeloblastic leukemia; CAR-T: chimeric antigen receptor-T cells; CLL: chronic lymphoid leukemia; Fv: variable fragment; HCL: hairy cell leukemia; FL: follicular lymphoma; LCL: large cell lymphoma; NHL: non-Hodgkin lymphoma; scFv: single-chain variable fragment; SLE: systemic lupus erythematosus; N/A: not apply.