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Siglecs as Immune Cell Checkpoints in Disease

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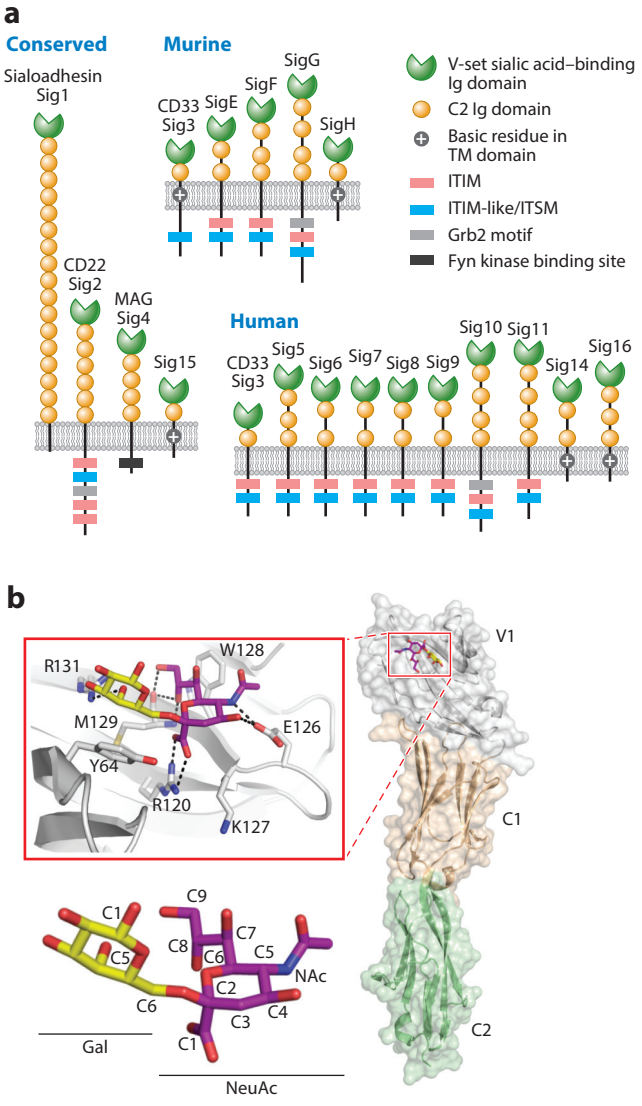
Abstract

Sialic acid–binding immunoglobulin-type lectins (Siglecs) are expressed on the majority of white blood cells of the immune system and play critical roles in immune cell signaling. Through recognition of sialic acid–containing glycans as ligands, they help the immune system distinguish between self and nonself. Because of their restricted cell type expression and roles as checkpoints in immune cell responses in human diseases such as cancer, asthma, allergy, neurodegeneration, and autoimmune diseases they have gained attention as targets for therapeutic interventions. In this review we describe the Siglec family, its roles in regulation of immune cell signaling, current efforts to define its roles in disease processes, and approaches to target Siglecs for treatment of human disease.

INTRODUCTION

The sialic acid–binding immunoglobulin (Ig)-type lectins (Siglecs) are found on most white blood cells of the immune system and have in common an N-terminal Ig domain that recognizes sialic acid–containing glycans commonly found on glycoproteins and glycolipids (1, 2) (**Figure 1*a*,*b***).



c

Conserved

Name	Expression	Ligand
Sig1	Mac, DC	α3 β4
Sig2	B, cDC, MC	α6 β4
		α6 β4 6S
Sig4	OD, Schw	α3 β3
Sig15	Ocl, Mac	α6

Human

Name	Expression	Ligand
Sig3	Mac, MyP, Mo, Mic	α6 β4
Sig5	N, Mo, B, MC	α6
		α8 α3
Sig6	B, MC, Troph	α6
Sig7	NK, Mo, T, MC	α8 α3
Sig8	Eo, Ba, MC	6S α3 β4
Sig9	Mo, N, cDC, NK, T	6S α3 β4 α3
Sig10	B, Mo, Eo	α6 β4
Sig11	Mac, Mic	α8 α3
Sig14	N, Mo	α6
		α8 α3
Sig16	Mac, Mic	α8 α3

Murine

Name	Expression	Ligand
Sig3	Mac, Mic	α6 β4
SigE	N, Mo, cDC, Mic	α3 β3 α6
SigF	Eo, Mac	α3 β4
		6S α3 β4
SigG	B, cDC	α3 β4
		α6 β4
SigH	pDC, Mac, Mic	Not determined

NeuAc NeuGc Gal Fuc
GalNAc GlcNAc S Sulfate

(Caption appears on following page)

Figure 1 (*Figure appears on preceding page*)

Human and murine Siglecs. (*a*) Structural features of functional human and murine Siglecs, including four members that are conserved in all mammals, and so-called CD33-related Siglecs numbering five in mice and ten in humans (1, 2, 193). Each Siglec (Sig) has an N-terminal V-set immunoglobulin (Ig) domain that contains the conserved sialic acid–binding site and 1–16 C2 Ig domains. On the cytoplasmic side most Siglecs exhibit characteristic regulatory motifs including immunoreceptor tyrosine inhibitory motif (ITIM), ITIM-like, immunoreceptor tyrosine switch motif (ITSM), growth factor receptor-bound 2 (Grb2) motif, and a Fyn kinase binding site. Several other Siglecs contain positively charged amino acid residues in the transmembrane domain that can associate with activating adaptor proteins such as DAP12 with an immunoreceptor tyrosine activation motif (ITAM) (1, 2, 193, 224). (*b*) Crystal structure of a portion of human CD22 including the N-terminal V-set and two C-set Ig domains (*right*). An expanded view of the sialic acid–binding site with a bound ligand fragment (NeuAcα2–6Gal) shows interaction of the C-1 carboxyl group of the sialic acid with the conserved arginine (R120) found in all Siglecs (18). (*c*) Shown for each Siglec is its cell type expression and preferred natural sialoside ligand(s) (1, 35). Cell types are mainly white blood cells in the immune system, including B cells (B), basophils (Ba), conventional and plasmacytoid dendritic cells (cDC and pDC), Eosinophils (Eo), macrophages (Mac), mast cells (MC), microglia (Mic), monocytes (Mo), natural killer cells (NK), neutrophils (N), osteoclasts (Ocl), and T cells (T), and a few cell types outside the immune system such as oligodendrocytes (OD), Schwann cells (Sch), and placental trophoblasts (Troph). All Siglecs except Siglec-H are known to bind terminal sequences on glycans of glycoproteins and glycolipids, with some having high sequence specificity for their ligands (e.g., Sig2, Sig7, Sig8), while others exhibit a broader specificity (1). Abbreviations: MyP, myeloid progenitor; TM, transmembrane.

Most Siglecs have regulatory motifs in their cytoplasmic domains that participate in regulation of cell signaling (1–4). Since sialic acids are found on all mammalian cells, Siglecs can help immune cells distinguish between self and nonself and serve as immune checkpoints to prevent unwanted immune responses (1, 2, 5). While the diverse roles of Siglecs in immune cell functions are beginning to be elucidated, the fact that they are expressed on most immune cells positions them to participate in highly diverse cellular immune responses that are both beneficial and harmful. Moreover, as coreceptors that regulate cell signaling, they are increasingly recognized as targets for development of strategies to augment or suppress immune cell responses for therapeutic benefit in numerous diseases (1, 3, 5–9).

In this review we describe the diverse Siglec family with respect to their overall structure, cellular distribution, recognition of glycan ligands, and regulation of cell signaling as immune checkpoints in health and disease. Special emphasis is placed on understanding the critical role of microdomain localization of the Siglecs with regard to their interaction with glycan ligands and as coreceptors for signaling receptors. Although there is a growing literature on the roles of Siglec-mediated immune responses to commensal and pathogenic microorganisms, this work is only mentioned in selected contexts, and the reader is referred to other recent work on the topic (10–14). Finally, we summarize the roles of Siglecs in immune cell–mediated disease and emerging strategies to target Siglecs and modulate their functions for treatment of disease.

THE SIGLEC FAMILY

Structure and Topology

The Siglecs are a subfamily of the Ig superfamily comprised of a single N-terminal V-set Ig domain that binds sialic acid–containing glycans, and 1 to 16 C-set Ig domains. These Ig domains are structurally analogous to the variable (V) and constant (C) Ig domains of an antibody, respectively. As illustrated in **Figure 1*a***, of the 14 human and 9 murine Siglecs, 4 are highly homologous, sialoadhesin (CD169, Siglec-1), CD22 (Siglec-2), myelin-associated glycoprotein (MAG, Siglec-4), and Siglec-15, which are clear orthologs in structure and function in all mammals. The remaining Siglecs are called CD33-related Siglecs since they are considered to have evolved from

duplication of the CD33 gene (2). Human Siglecs are numbered numerically, according to the order in which they were discovered, and murine Siglecs not homologous to human Siglecs are given alphabetic designations (e.g., Siglec-E-H). Missing are human Siglec-12 and -13, which are nonfunctional in humans (1, 2). While CD33-related Siglecs in humans and mice are not strict orthologs, some are related by cell type expression, ligand specificity, and regulatory functions, as discussed further below.

There are now crystal structures or nuclear magnetic resonance structures reported for Siglec-1 (sialoadhesin) (15–17), Siglec-2 (CD22) (18), Siglec-3 (CD33) (19), Siglec-5 (20), Siglec-7 (21), and Siglec-8 (22). All structures contain the V-set domain, either alone or with one or two C-set domains. The sialic acid–binding site is a shallow pocket in the V-set domain, as shown for the V-set domain of CD22 (**Figure 1*c***), which has been validated for several Siglecs by direct cocrystallization with their glycan ligands. Sialic acid is a nine-carbon sugar with a carboxyl group at C1; the anomeric carbon at C-2, which is linked to the next monosaccharide in the glycan; an *N*-acetyl group at C-5; and a polyhydroxy side chain formed by C7–C9. The sialic acid–binding sites of all Siglecs contain a conserved positively charged arginine that interacts with the negatively charged C-1 carboxyl group on the sialic acid. The structure for CD22 comprising the first three Ig domains shows that the V-set domain tilts at a 120° angle from the two C-set domains. Combining this information with negative stained electron microscopy images of the full-length CD22 reveals that it is a fairly rigid rod projecting the V-set domain away from the cell membrane (18).

Most Siglecs contain structural features of receptors involved in cell signaling (**Figure 1*a***), with the only exception being sialoadhesin/Siglec-1. The majority contain one or more consensus immunoreceptor tyrosine inhibitory motifs including a classic immunoreceptor tyrosine inhibitory motif (ITIM) (I/V/LxYxxL/V), an ITIM-like motif (D/E xYxEV/IK/R), or an ITSM switch motif (TxYxxV/I) that can in principle participate in inhibitory or activating signals (4, 23). As further described below, ITIMs of inhibitory receptors are phosphorylated by Src kinases (e.g., Lyn) and recruit the Src-homology 2 domain (SH2)-containing phosphatases SHP-1 and SHP-2 that dephosphorylate the signaling molecules in the activation complex and suppress signaling (4, 24, 25). In addition to ITIM and ITIM-like motifs, several Siglecs contain other regulatory sites in the cytoplasmic domain including a motif for binding Grb2 in B cell Siglecs CD22, Siglec-G, and Siglec-10 (4, 25), and a Fyn kinase binding site in MAG/Siglec-4 that plays a key role in signaling required for normal myelin formation (26).

In contrast to the inhibitory Siglecs, Siglecs-14, -15, -16 and -H are considered to be activating Siglecs. They have a minimal cytoplasmic domain with no tyrosine regulatory motifs, but they have positively charged amino acid residues in their transmembrane domain that associate with the coreceptor DAP12 (4, 11, 27–31). While DAP12 has no extracellular receptor domain, it contains an immunoreceptor tyrosine activation motif (ITAM) in its cytoplasmic domain. Thus, when bound to DAP12 these Siglecs are effectively activating receptors, with the Siglec providing the ligand-binding function, and DAP12 the signaling function. Each of these Siglecs has been demonstrated to associate with DAP12 and/or elicit an activating activity (4, 11, 27–31).

Siglec-14 and Siglec-16 are somewhat unique since they appear to have evolved through gene duplication of inhibitory Siglec-5 and -14, respectively (32–34). Indeed, in their extracellular domains Siglec-5 is highly homologous to Siglec-14, and Siglec-11 is highly homologous to Siglec-16. Moreover, these paired receptors, Siglec-5 and Siglec-14 and Siglec-11 and Siglec-16, are typically expressed together. Evidence suggests that the paired activating Siglecs evolved in response to pathogens that cloak themselves in sialic acid–containing glycans that can exploit the inhibitory Siglecs to suppress immune attack. With the paired Siglec activating response, an immune response to the sialylated pathogen can be activated (11, 31–34).

Cell Type Expression and Glycan Ligands

The functions of the Siglecs are tied to the cell type they are expressed on, ligands that they recognize, the regulatory motifs/activities they carry, and their subcellular localization relative to other receptors involved in the immune response. As mentioned above, with few exceptions members of the Siglec family are expressed on several cell types that comprise the immune system. Only a few Siglecs are expressed predominately on one cell type, such as sialoadhesin (CD169; Siglec-1) on macrophages, CD22 on B cells, and Siglec-8 on eosinophils, but these Siglecs have also been detected at low levels on other cell types (**Figure 1*c***).

Although the Siglecs typically bind a range of sialic acid–containing glycans as ligands with overlapping specificity, they each exhibit a unique specificity profile and exhibit different preferences for sialic acid–containing glycans as illustrated in **Figure 1*c*** (1, 3, 35). Although much has been learned about the roles of Siglecs in immune cell signaling and their impact on disease processes, relatively little is known about their natural ligands in the context of cell-to-cell interactions.

The most extensively studied for its ligand-binding specificity is CD22 (Siglec-2). The differences in ligand specificity of human and murine CD22, a conserved Siglec, and changes in ligand expression during B cell differentiation exemplify the subtleties that define biologically important Siglec-ligand interactions in these two species. CD22 is strongly conserved in mammals and is well recognized for its function as a regulator of B cell receptor (BCR) signaling (1, 4, 25, 36, 37). One well-known difference between the human and murine glycome relevant to CD22 ligands is that mice have both 5-*N*-acetyl-neuraminic acid (NeuAc) and 5-*N*-glycollyl-neuraminic acid (NeuGc), while humans have lost the ability to produce NeuGc and have only NeuAc (38). Murine CD22 has evolved a strict specificity for glycans terminating in the sequence NeuGcα2–6Galβ1–4GlcNAc, and it binds only weakly to the same sequence terminating with NeuAc (35). Cell surface glycans of murine B cell glycoproteins have high levels of the NeuGcα2–6Galβ1–4GlcNAc sequence that bind to CD22 as *cis*-ligands, masking the CD22 ligand-binding site (39). Remarkably, following BCR ligation, proliferating B cells downregulate the hydroxylase that converts NeuAc to NeuGc, producing the sequence NeuAcα2–6Galβ1–4GlcNAc detected by the antibody G7, commonly used to detect germinal center B cells. Since CD22 requires NeuGc, this change effectively results in loss of *cis*-ligands and unmasking CD22 (40, 41).

Since humans cannot produce NeuGc the situation for human CD22 is different, but the functional paradigm is recapitulated. For human CD22 the highest-affinity ligand found on B cells is a sulfated sialoside NeuAcα2–6Galβ1–4[6-SO_4]GlcNAc (40, 42). Upon activation of B cells, the sulfotransferase involved in its biosynthesis is downregulated, resulting in B cells that have the lower-affinity NeuAcα2–6Galβ1–4GlcNAc. Thus, CD22 in the two species has evolved to recognize a high-affinity *cis*-ligand on B cells unique to that species, and in both cases activation of B cells results in a biosynthetic change to produce a lower-avidity ligand on the proliferating B cells. While the biological implication of this dramatic reduction of CD22 ligands in germinal center B cells is not yet fully understood, B cells of transgenic mice with no *cis*-ligands or with CD22 mutations that inactivate ligand binding exhibit increased association of CD22 with the BCR and hypoproliferation upon BCR ligation (37, 43, 44).

Another example of a human and murine Siglec pair with high specificity for their ligands is Siglec-8 and its murine paralog Siglec-F on eosinophils. These two Siglecs are unique in their recognition of the sulfated sialoside NeuAcα2–3[6SO_4]Galβ1–4GlcNAc as a preferred ligand (45–49). However, Siglec-8 appears to be highly specific for the 6-sulfate group on the same galactose as the sialic acid, while Siglec-F exhibits a broader specificity for other α2–3 sialosides without the sulfate group (46, 48, 49). This unique sulfated sialoside sequence has recently been found on

high-molecular-weight sialylated keratan sulfate proteoglycans in human lung tissue, which places them in a favorable context for interaction with Siglec-8 on eosinophils (49).

Most other Siglecs bind to natural sialosides with very low avidity and with broad overlaps in their specificity. This has motivated several groups to develop synthetic glycan ligands of Siglecs with high specificity and avidity to target them and study their functions (16, 17, 50–55). The general approach has been to introduce unnatural substituents at the 5-C and 9-C positions of sialic acid that impart additional specificity and affinity through their interactions with Siglecs in nooks and crannies around the conserved ligand-binding pocket (**Figure 1*b***). Through these efforts there are now many examples of synthetic glycan ligands that can be used to investigate functions of and exploit the signaling properties of individual Siglecs in complex biological systems.

SIGLECS AS ENDOCYTIC RECEPTORS

Most Siglecs are also endocytic receptors that can carry cargo from the cell surface into intracellular compartments such as endosomes. CD22 was identified as an endocytic receptor several years before it was identified as a member of the Siglec receptor family (56). Subsequently, most human and murine Siglecs have been demonstrated to be endocytic/phagocytic receptors, including Siglecs 1–5, 7–10, and E–H (57–68). While the relevance of endocytosis to the biology of most Siglecs is still under investigation, analysis of endocytic functions of several exemplary Siglecs have revealed both similarities and differences in the endocytic mechanisms of this family.

Sialoadhesin (Siglec-1/CD169) is expressed on sinusoidal macrophages and some peripheral dendritic cells that capture antigen and present it to the adaptive immune system. While sialoadhesin has no signaling motif, ligation with antibody causes endocytosis in a clathrin/dynamin-dependent manner, indicative of endocytosis mediated by clathrin-coated vesicles that traffic between the cell surface and early endosomes (58, 69). Sialoadhesin-mediated endocytosis has been directly implicated in the capture, dissemination, and/or infection of several membrane-enveloped viruses, including (70) HIV and Ebola virus (69, 71, 72) (**Figure 2*a***). As discussed further below, the endocytic capacity of sialoadhesin offers the potential to exploit it as a target for delivery of antigens to macrophages to boost or alter an immune response (58, 73–75).

As the first Siglec to be identified as an endocytic receptor, CD22 is expressed predominately on B cells and to a lesser extent on mast cells and dendritic cells, and it is known as a negative regulator of BCR signaling (1, 4, 25, 76–79). Although endocytosis of antibody bound to CD22 was initially concluded to result in transport of the internalized complex to lysosomes for degradation (65), it was later shown that CD22 constitutively recycles between the cell surface and early endosomes (43, 62, 66), and slowly over time, a portion of the complex is shuttled to and degraded in lysosomes (62, 65). In a typical experiment, antibody is bound to CD22 at 4°C, and upon warming to 37°C it disappears from the cell surface. While it is tempting to conclude that antibody ligation induces endocytosis of CD22, in some cases in the presence of excess antibody a net shift of CD22 from intracellular pools to the cell surface was observed (62). Once equilibrated with the intracellular pool of CD22, the antibody-CD22 complex remains stable in the acidic endosomes and recycles back to the surface of the cell. In contrast to antibody, multivalent ligands and ligand-decorated nanoparticles endocytosed by CD22 are released inside the cell as endosomes are acidified, and by recycling back to the cell surface without ligand, CD22 is able to shuttle additional ligand into the endosomal compartments (62, 80). The results underscore the fact that CD22 undergoes constitutive endocytosis, and contrary to what is often assumed antibody ligation may shift the equilibrium to a have a greater proportion of CD22 on the surface of the cell.

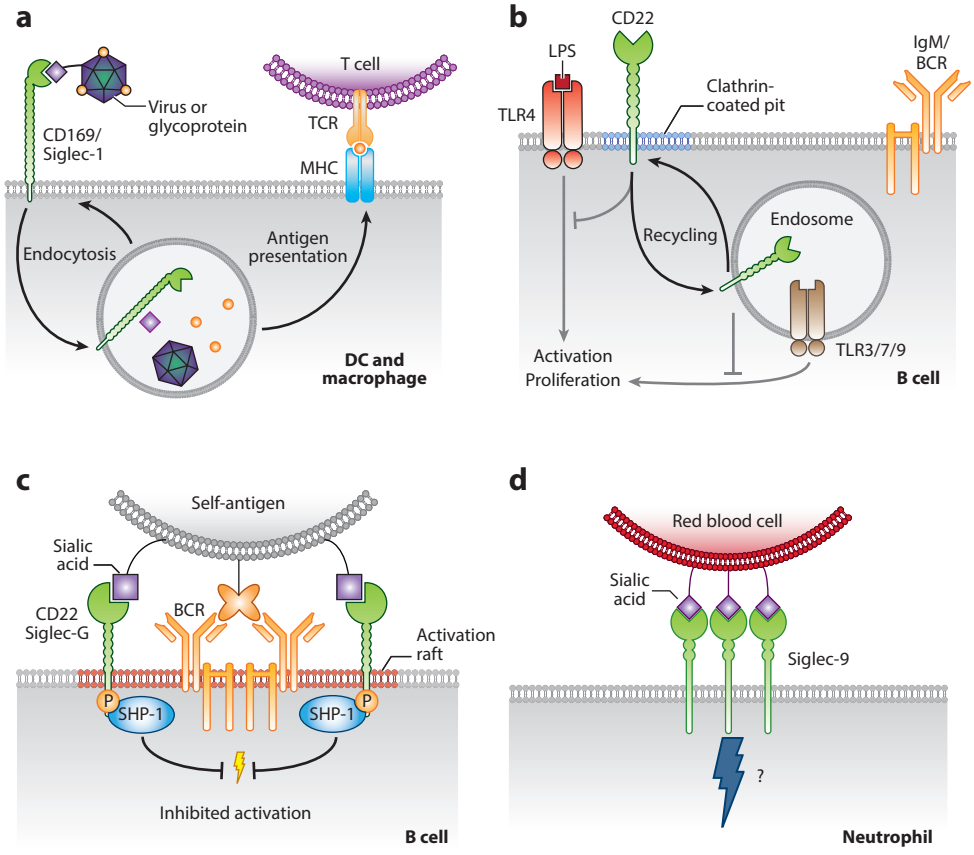


Figure 2

Endocytosis and signaling functions of Siglecs are tied to their roles in immune cell responses. (*a*) Most Siglecs are endocytic receptors, as illustrated for sialoadhesin/Siglec-1/CD169 on macrophages, known for endocytosis of sialic acid–containing antigen to endosomes for processing and presentation via the major histocompatibility complex (MHC) to T cells (58, 73, 225, 226). (*b*) In B cells, CD22 is a recycling endocytic receptor distributed between cell surface and endosomal compartments shared with Toll-like receptors (TLRs), resulting in strong constitutive suppression of TLR signaling (62, 106, 107). (*c*) While B cell Siglecs, CD22 and Siglec-G, are not constitutively colocalized with the B cell receptor (BCR), ligand-mediated recruitment to an immunological synapse with a membrane antigen on another cell results in strong suppression of BCR signaling (43, 44, 110, 111). (*d*) Negative signaling of Siglec-9 on neutrophils resulting from ligation via *trans*-ligands on red blood cells, resulting in suppression of activation and apoptosis of neutrophils in blood (134). Abbreviations: DC, dendritic cell; LPS, lipopolysaccharide; TCR, T cell receptor.

CD22 exhibits a clathrin/dynamin-dependent endocytic mechanism that phenocopies the classic recycling of the transferrin receptor, differing primarily in kinetics of internalization (66, 81). Several different motifs in the cytoplasmic domain have shown to be important for endocytosis. The two distal ITIMs interact with the clathrin adaptor protein A50, and when these ITIMs are mutated endocytic efficiency is dramatically reduced (62, 81). A second membrane-proximal motif (R737/Q739 in mCD22) is equally important for endocytosis and appears to work synergistically with the ITIMs. Mutation of either set of motifs reduces endocytosis by over 50-fold (62, 82). The endocytic mechanism and resulting subcellular localization of CD22 also play a critical role in CD22 regulation of TLR and BCR signaling (**Figure 2*b*,*c***), as discussed further below.

While both CD22 and sialoadhesin are clathrin/dynamin-dependent endocytic receptors, sialoadhesin has no ITIMs in its cytoplasmic domain, so clearly they differ in the precise manner in which they associate with clathrin-rich domains (58, 66, 81). Several other inhibitory Siglecs have also been demonstrated to undergo endocytosis through a clathrin-dependent mechanism, including MAG (Siglec-4) and Siglec-8 (63, 68). In contrast, however, Siglec-F undergoes endocytosis in a clathrin/dynamin- and caveolin-independent mechanism that is dependent on ADP-ribosylation factor 6 (ARF6), a small GTPase associated with a less-studied endocytic pathway (66, 83, 84). Although human Siglec-8 and murine Siglec-F are considered to be paralogs on eosinophils (46), the facts that Siglec-8 endocytosis is primarily clathrin dependent and Siglec-F endocytosis is clathrin independent show that their functions have diverged (63, 66).

In summary, while most if not all Siglecs are endocytic receptors, no two Siglecs have been demonstrated to have identical endocytic mechanisms for those that have been studied in detail. Little studied to date is the connection between the role of Siglecs as endocytic receptors and their functions as checkpoints in immune cell signaling.

SIGLECS IN IMMUNE CELL SIGNALING

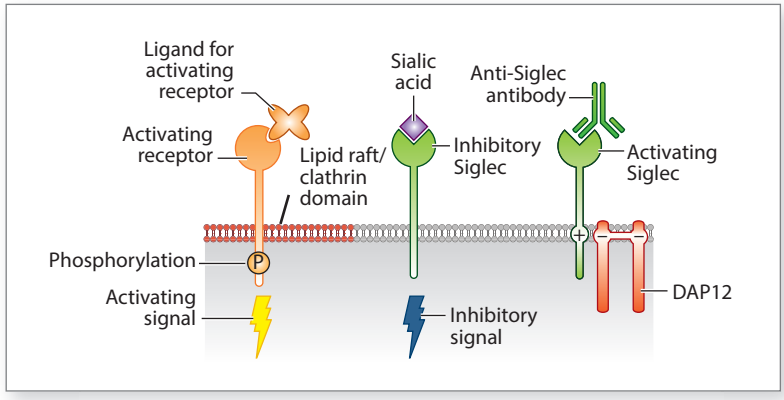
Siglecs serve as checkpoints in immune cell signaling in diverse contexts through their roles as inhibitory coreceptors and as activating receptors. Their signaling functions are influenced by their microdomain localization and their interactions with ligands on the same cell (*cis*-ligands) and other cells (*trans*-ligands) (1, 3, 4, 10, 25). As endocytic receptors, most Siglecs are also present both on the cell surface and in endosomal compartments (58, 60, 62, 63, 66, 85, 86). Moreover, since Toll-like receptors (TLRs) are also found in endosomal compartments, and cell surface receptors are often endocytosed once ligated, the roles of Siglecs in cell signaling are relevant to their locations in both cell surface and endosomal compartments (87–90). In this section we briefly discuss the importance of microdomain localization for understanding the roles of Siglecs on signaling. We then describe the impact of Siglecs on signaling for several cell types that have been studied in detail.

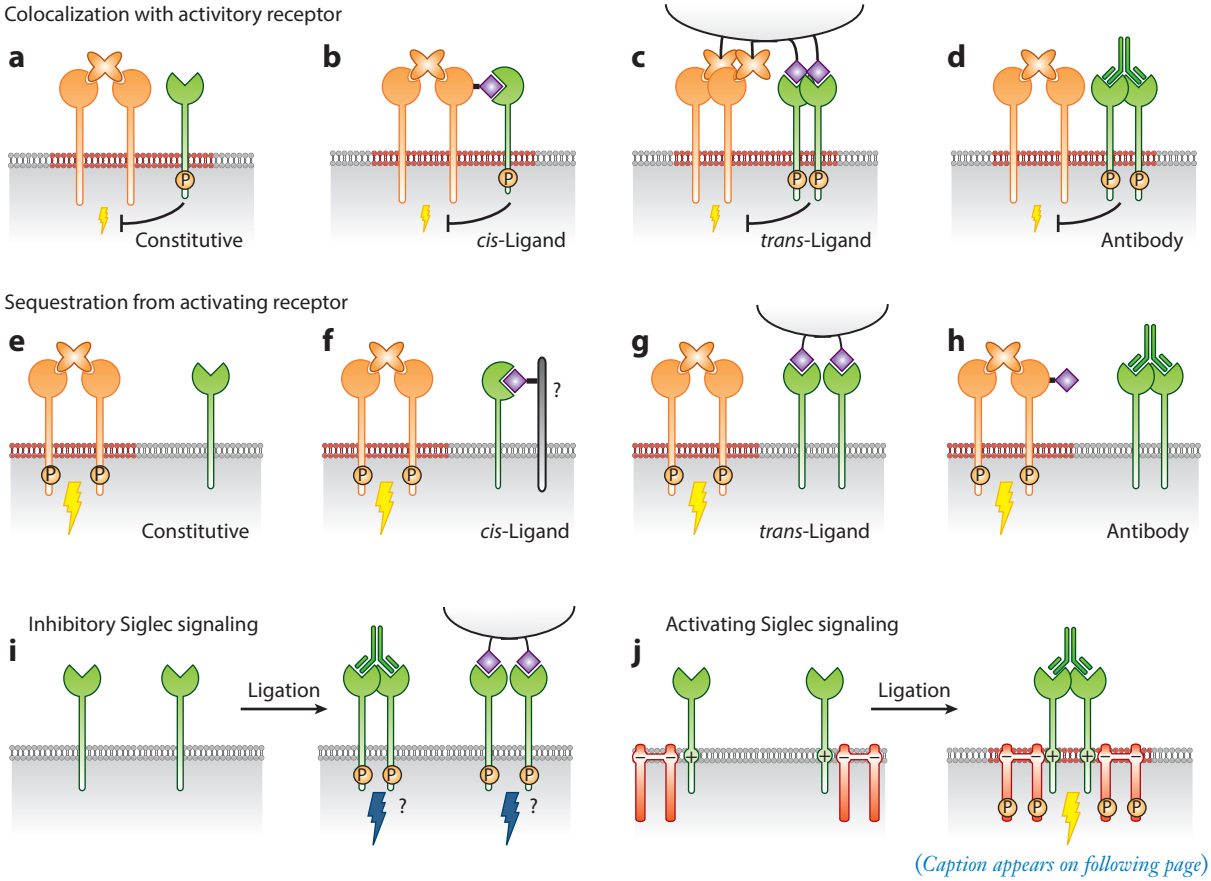
Microdomain Localization

A critical but often overlooked aspect of Siglec function is the microdomain localization of the Siglec relative to that of other cellular activation complexes that are themselves localized in microdomains, which may be referred to as nanodomains, lipid rafts, caveolae, and/or clathrin domains (88, 91–95). For some inhibitory Siglecs like CD22, phosphorylation and subsequent recruitment of SHP-1 requires activated kinases localized to the activation complex (1, 4, 25, 96–98). As illustrated in **Figure 3*a–d***, colocalization of an inhibitory Siglec with an activating receptor can occur constitutively, be enforced by *cis*- or *trans*-ligand interactions, or result from antibody-mediated relocalization to the activation domain. Conversely, the Siglec may be sequestered away from the activating receptor (**Figure 3*e–b***), as a result of being in a microdomain remote from the signaling complex, sequestered by interactions with *cis*- or *trans*-ligands, or sequestered by antibody ligation. Inhibitory activities observed for Siglecs upon exposure to anti-Siglec antibodies or multivalent *trans*-ligands have suggested that ligation of inhibitory Siglecs can activate/phosphorylate the ITIMs and produce a negative signal (**Figure 3*i***), although the underlying mechanism has not yet been defined. Activating Siglecs associate with the DAP12 that has an ITAM motif and can activate signaling when ligated with an anti-Siglec antibody (4, 11, 27–31) (**Figure 3*j***). In the following sections particular attention is paid to the impact of microdomain localization in the diverse cellular contexts that Siglecs can impact immune cell responses.

B Cell Signaling

Siglecs have been most extensively studied as regulators of signaling receptors in B cells. B cells contain two Siglecs, CD22 and Siglec-10 in humans, and CD22 and Siglec-G, an ortholog of Siglec-10, in mice. CD22 was known as a regulator of BCR signaling before it was identified





(Caption appears on following page)

Figure 3 (*Figure appears on preceding page*)

Critical role of microdomain localization of Siglecs as checkpoints in immune cell signaling. (*a–h*) Most Siglecs are inhibitory coreceptors that modulate the activity of activating receptors (1–4). (*a–d*) In this context, the microdomain localization of the Siglec relative to the activating receptor plays a major role in its regulation of that receptor. Colocalization of the Siglec with the activating receptor (*a*) constitutively, or mediated (*b*) by *cis*-ligand binding, (*c*) by *trans*-ligand binding, or (*d*) by antibody-induced relocalization resulting in activation phosphorylation of immunoreceptor tyrosine inhibitory motifs (ITIMs) by locally activated kinases, and recruitment of phosphatases (e.g., SHP-1, SHP-2), which in turn suppress cell signaling by dephosphorylation of signaling molecules. (*e–h*) Conversely, sequestration of the Siglec away from activation rafts (*e*) by constitutive localization to another domain, (*f*) by *cis*-ligand binding, (*g*) by *trans*-ligand binding, or (*h*) by antibody-induced relocalization preventing physical association with the locally activated kinases, phosphorylation, recruitment of phosphatases, and regulation of the activating receptor. (*i*) In principle, ligation of inhibitory Siglecs by antibodies or ligands could directly cause activation of kinases that phosphorylate regulatory motifs, recruit phosphatases, and suppress signaling pathways (124, 134). (*j*) For Siglecs that associate with DAP12 through a positive charge in their transmembrane domain, ligation can induce kinase activation and initiate an activating signal (27, 29, 30).

as a sialic acid–binding receptor (99, 100). Its role as a negative regulator of BCR signaling was documented by comparing anti-IgM-induced activation of B cells from wild-type and CD22-deficient mice, where CD22 null cells showed hyperresponsiveness in activation-induced Ca^{++} mobilization and proliferation (101–103). CD22 inhibition of signaling induced by BCRs involves phosphorylation of two distal ITIMs by locally activated Lyn, recruitment of SHP-1 phosphatase, and dephosphorylation of the BCR receptor complex and downstream signaling molecules (1, 25). The importance of BCR-induced activation of Lyn in phosphorylation of CD22 is underscored by the fact that CD22 ligation itself does not suppress BCR signaling. Indeed, ligation of CD22 resulting from contact of B cells with anti-CD22-coated beads sequesters CD22 away from the BCR, causing hyperresponsiveness to activation by ligation with anti-IgM, while coligating CD22 to the BCR with beads containing both anti-CD22 and anti-IgM causes inhibition (104).

Several studies suggest that CD22 regulation of BCR signaling is context dependent, involving its microdomain localization and glycan ligand interactions. On a resting B cell, the BCR and CD22 are only weakly associated, with the BCR and CD22 localized to separate partially overlapping nanoclusters, each occupying approximately 10% of the total surface of the cell (43, 44, 91) (e.g., **Figure 3*e***). The majority of CD22 is localized to clathrin-coated pits (**Figure 2*b***), forming clusters resulting from *cis*-ligand interactions of CD22 with sialic acids on glycans on adjacent CD22 molecules (43, 44, 105). The role of *cis*-ligand interactions in sequestering CD22 from IgM is supported by analysis of B cells deficient in the ST6Gal 1 sialyltransferase that synthesizes CD22 ligands, and B cells with a CD22 mutation removing the conserved arginine required for sialic acid binding (R130E) (24, 43, 44). In both cases, disruption of CD22 *cis*-ligand interactions results in a greater association with IgM and stronger suppression of B cell activation.

While CD22 is mainly viewed as a coreceptor of the BCR, CD22 also regulates TLR signaling (106, 107). B cells from $CD22^{-/-}$ mice exhibited modest hypersensitivity to BCR activation with anti-IgM but showed profound increases in sensitivity to the ligands of TLR3, TLR4, and TLR9 (106, 107). The dramatic suppression of TLR signaling in wild-type B cells likely reflects the constitutive localization of CD22 in microdomains where these TLRs reside (**Figure 2*b***). There are also indications that CD22 plays activating roles in some contexts (103). In this regard, calcium signaling mediated by the B cell plasma membrane calcium ATPase has been demonstrated to involve an ITIM/SHP-1-independent signaling pathway mediated by the CD22 Grb2 tyrosine motif (108, 109).

In contrast to the case with soluble antigens or anti-IgM, CD22 strongly suppresses BCR activation of membrane antigens. Although *cis*-ligands impact the membrane and effectively limit the association of CD22 with the BCR, they do not prevent CD22 from interacting with *trans*-ligands on an adjacent cell, causing it to redistribute to the site of cell contact (110–112) (**Figure 2*c***). The structure of CD22 as a rigid rod with the ligand-binding site extended away from the membrane

makes it favorably positioned for *trans*-ligand-mediated recruitment to the immunological synapse with a membrane antigen (18) (**Figure 1*b***), resulting in profound suppression of B cell signaling that can lead to apoptosis of the impacted B cells (110, 111, 113). In this regard, it is notable that murine red blood cells do not express glycan ligands of either CD22 or Siglec-G, and membrane antigens on these cells robustly activate B cells (114).

Siglec-G is the other major Siglec on murine B cells with Siglec-10 as its ortholog on human B cells. Although regulation of BCR signaling by these Siglecs has not been studied in as much detail as that by CD22, they also suppress signaling through a similar mechanism that results in dephosphorylation of downstream signaling molecules (e.g., Erk, Akt) (36, 115–117). Although they are to some extent redundant in their functions, they do exhibit some clear differences. While both CD22 and Siglec-G are expressed on all B cells, Siglec-G is dominant on B1 cells and CD22 is dominant on B2 cells (36, 115–117). Moreover, CD22 and Siglec-G/10 differ in their ligand-binding preferences, with CD22 having high specificity for glycans terminating in the NeuGcα2–6Gal linkage, and Siglec-G binding both NeuGcα2–6Gal and NeuGcα2–3Gal glycans (98, 117). Moreover, in contrast to the ligand-mediated sequestration of CD22 from the BCR on resting B cells, Siglec-G exhibits ligand-mediated colocalization with the BCR on B1 cells (36, 115, 116).

Eosinophil Signaling

Eosinophils are key effector cells in asthma and allergic lung inflammatory diseases. Siglec-8 is selectively expressed on human eosinophils and mast cells, and weakly on basophils, and has been investigated as a therapeutic target in eosinophil-mediated diseases (3). Antibodies to Siglec-8 induce eosinophil apoptosis by a mechanism involving production of reactive oxygen species (ROS) and induction of caspase-3 (118–121). This apoptotic activity is dramatically enhanced by IL-5, GM-CSF, or IL-33. While the mechanism of anti-Siglec-8 induction of apoptosis is still under investigation, apoptosis appears to result from activation of an Akt/p38/JNK-1 pathway, and amplification of apoptosis by IL-5 or GM-CSF appears to result from upregulation of integrins that increase cell adhesiveness and susceptibility to apoptosis (118, 121). In this context, Siglec-8 appears to be an activating receptor (118, 121). In this regard, Siglec-8 is reported to have two isoforms in eosinophils (120), a longer isoform with an extended cytoplasmic domain with two ITIMs (**Figure 1*a***) and a more abundant short isoform with a truncated cytoplasmic domain with no ITIMs (120, 122, 123). It remains to be determined if one or both isoforms mediate anti-Siglec-8 activation of the JNK-1 pathway and apoptosis of eosinophils. In this regard, Siglec-7 is also expressed on eosinophils, and while anti-Siglec-7 suppresses activation and production of cytokines characteristic of an inhibitory receptor, it causes no apoptosis of GM-CSF-treated cells (124) (**Figure 1*a***), consistent with anti-Siglec-induced apoptosis affecting its ITIMs.

Signaling in Other Immune Cells

Siglecs also modulate signaling in other immune cells in numerous other contexts. Liu and coworkers have described a Siglec-G/10-CD24 axis for regulation of damage-associated molecular pattern (DAMP) receptors in human and murine dendritic cells and murine T cells (125). Regulation involves *cis*-ligand-mediated association of the Siglec and CD24, which in turn associates with DAMP receptors for negative regulation of signaling to control the damage response (1, 125–127). TLRs, as exemplary pathogen-associated molecular pattern (PAMP) receptors on dendritic cells, have also been reported to be negatively regulated by Siglec-7 and -9, and in a ligand-dependent manner (128). Similarly Siglec-E has been reported to regulate TLR4 by Siglec-E on murine macrophages (129, 130), but this result has not been confirmed by others (61). CD33 (Siglec-3) has been reported to negatively regulate the NKG2D/DAP10 activating receptors in NK cells

(131), and Siglecs-7 and -9 to be negative regulators of NK-mediated cell killing of tumor cells expressing ligands of these Siglecs (132, 133).

A common perception is that ligation of Siglecs can send a negative signal to the cell (e.g., **Figure 3*i***) (1). Notable examples are erythrocyte sialic acids engaging Siglec-9 on neutrophils to suppress activation and apoptosis (134) (**Figure 2*d***), sialylated glycans on group B *Streptococcus* engaging Siglec-9 to suppress platelet-mediated killing (13), and anti-Siglec-7-mediated suppression of NK cell activation and proliferation (133, 135). However, these contexts have not been investigated in sufficient detail to determine whether Siglec ligation itself can induce kinase activation, ITIM phosphorylation, and recruitment of SHP-1/SHP-2 phosphatases, or whether ligation results in altered microdomain localization to place the Siglec in the context of kinases activated by other receptors (e.g., **Figure 3*c*,*d***). This is an area that requires more attention to better understand the roles of Siglecs in regulation of cell signaling.

As mentioned above, all the activating Siglecs that have a positive charge in their membrane domains (Siglec-14, -15, -16, and -H) have been shown to associate with DAP12 that contains ITAM motifs in its cytoplasmic domain (4, 11, 27–31). In multinucleated osteoclasts, Siglec-15 is constitutively associated with DAP12, and anti-Siglec-15 ligation causes phosphorylation of DAP12, resulting in downstream phosphorylation of Akt and activation of the Akt pathway (136, 137). Subsequent to ligation, Siglec-15 was endocytosed and degraded in lysosomes (137). Analysis of Siglec-15 activity in macrophages has suggested that ligation of Siglec-15 with sialic acid–containing ligands on tumor cells also causes activation and downstream TGF-β secretion (30).

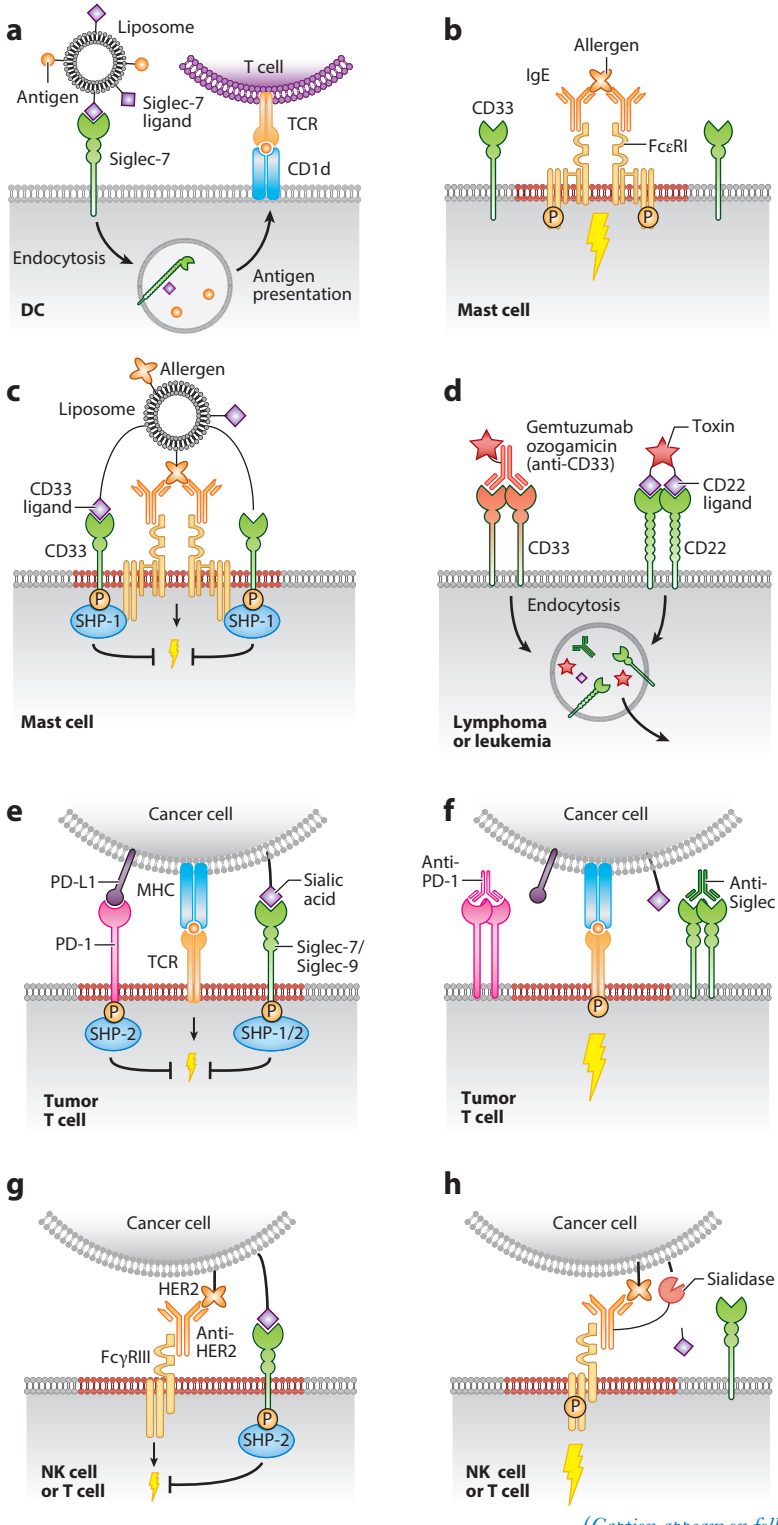
SIGLECS AS CHECKPOINTS IN DISEASE

Modulating Adaptive Immune Responses

The endocytic properties of Siglecs have been exploited for targeted delivery of antigen to key antigen-presenting cells (APCs) such as macrophages and dendritic cells to elicit desired immune responses (1, 9) (**Figures 2*a*** and **4*a***). Targeting CD169/sialoadhesin/Siglec-1-expressing macrophages and dendritic cells with protein/peptide antigens conjugated to a CD169 antibody or nanoparticles bearing a synthetic glycan ligand of CD169 (CD169L) has been shown to enhance a T cell immune response (73, 74, 138, 139), and to present intact antigen to B cells (75). Similarly, delivery of a glycolipid antigen (α-galactosyl-ceramide) via CD169L-liposomes induced CD1d-restricted activation of natural killer T (NKT) cells (86). CD169-targeted liposomes formulated with or without adjuvants have been shown to bias T cell immune responses to $CD4^+$ helper cells or $CD8^+$ cytotoxic T cells, which may have an implication in cancer immunotherapy (74). Finally, Siglec-7-targeted liposomes have been used to deliver mycobacterial antigen to dendritic cells for display on CD1b to promote CD1b-restricted activation of T cells that could improve efficiency of vaccination for mycobacteria (60) (**Figure 4*a***).

Autoimmune Disease and Immune Tolerance

Many autoimmune diseases such as rheumatoid arthritis, Grave disease, thrombotic thrombocytopenic purpura (TTP), and systemic lupus erythematosus (SLE) are based on a deficiency in normal mechanisms of immune tolerance, resulting in production of autoantibodies that result in inflammatory disease (140, 141). As regulators of immune cell signaling, Siglecs play a significant role in normal homeostasis and self-tolerance (25, 116, 142–145). Mice with deficiencies of the two major B cell Siglecs, CD22 and Siglec-G, develop autoimmune antibodies to nucleic acids (dsDNA/ssDNA/RNA) and protein (IgG-Fc/rheumatoid factor) (106, 107, 145). Interestingly, the nucleic acid antigens are ligands of TLRs, and both CD22 and Siglec-G are in microdomains that



(*Caption appears on following page*)

Figure 4 (*Figure appears on preceding page*)

Examples of targeting Siglecs to modulate immune cell responses in disease. (*a*) Delivery of antigens to antigen-presenting cells using liposomes with glycan ligands targeting endocytic Siglecs (60, 73). (*b*,*c*) Suppressing IgE-mediated mast cell responses to allergen. (*b*) Allergen-mediated ligation of the anti-allergen IgE-FcεRI complex activates mast cells leading to degranulation and anaphylaxis (227). (*c*) Recruitment of CD33 (Siglec-3) to the IgE-FcεRI complex using a liposome displaying both allergen and ligand for CD33 suppresses mast cell activation and desensitizes to subsequent allergen challenge (76). (*d*) Treating lymphoma/leukemia cells by targeting endocytic Siglecs with toxin conjugated to antibodies or glycan ligands (85, 174, 175). (*e*) Like programmed cell death protein 1 (PD-1), inhibitory Siglecs are checkpoint inhibitors that can suppress antigen-mediated activation and killing of cancer cells by tumor-infiltrating T cells (185, 196). (*f*) Analogous to anti-PD-1, anti-Siglec antibodies can in principle prevent *trans*-ligand-mediated recruitment of Siglecs to the T cell receptor (TCR) immunological synapse, allowing activation and killing of tumor cells (185, 196, 197). (*g*) Antibody-dependent cellular cytotoxicity (ADCC) mediated by natural killer (NK) cells and T cells through recognition of tumor-specific anti-HER2 antibody by the FcγRIII receptor is suppressed by recruitment of inhibitory Siglecs by *trans*-ligands on the cancer cell (200, 228). (*h*) Engineered HER2 with sialidase destroys sialic acid–containing ligands on the cancer cell resulting in activation of ADCC killing by preventing recruitment of inhibitory Siglecs (200, 228). Other abbreviations: DC, dendritic cell; MHC, major histocompatibility complex.

endogenously regulate TLRs (**Figure 2*b***). In this regard, for B cells that recognize nucleic acid antigens, the loss of the Siglecs likely results in a lower threshold for TLR activation contributing to autoantibody production (25, 106, 107). Mice with mutations in the sialic acid–binding sites of CD22 and Siglec-G do not produce autoantibodies to nucleic acids (37), suggesting that the presence of the ITIMs is sufficient to suppress BCR/TLR responses to these antigens. CD22 and Siglec-G have also been demonstrated to induce tolerance to membrane antigens through their recruitment to the BCR immunological synapse by *trans*-ligands on the APC (110, 111) (**Figure 2*c***). However, to date there has not been a systematic search for increased autoantibodies to membrane antigens in aged CD22 and Siglec-G double knockout mice (25, 144).

In mice, CD22 alleles have been linked to susceptibility to SLE-like disease, as thoroughly reviewed by Clark & Giltiay (25) and Mahajan & Pillai (144). Strain background makes a difference since CD22 deficiency in C57Bl/6 produces little or no autoantibodies or SLE-like disease, but in strains that predispose mice to autoimmune disease, CD22 deficiency results in higher anti-DNA antibodies and glomerulonephritis (25, 144, 146, 147). In humans polymorphisms of the CD22 gene have been associated with susceptibility to rheumatoid arthritis, SLE, and cutaneous systemic sclerosis, but as yet the role of CD22 in the etiology of these diseases is not confirmed (148–150). Pillai and coworkers identified an association of common autoimmune disorders in a European population with a defective sialic acid *O*-acetylesterase (SIAE) that removes 9-*O*-acetyl substituents on sialic acids (151). This 9-*O*-Ac substitution blocks binding by CD22, and transgenic mice with SIAE deficiency showed altered BCR regulation by CD22, suggesting a link between SIAE/CD22 and autoimmune disease in humans (25, 145, 151, 152).

Based on the roles of B cell Siglecs in maintaining tolerance, several groups have investigated the potential to exploit Siglecs to induce antigen-specific tolerance (1, 25, 153). In analogy to induction of tolerance to membrane antigens by ligands on the same cell recruiting Siglecs to the immunological synapse (111, 113) (**Figure 2*d***), the concept is to use antigen-bearing polymers or liposomal nanoparticles that display synthetic glycan ligands of CD22 or Siglec-G to recruit Siglecs to the BCR/antigen complex (98, 113, 117, 154). In in vivo models, recruitment of the Siglec strongly suppresses activation of B cells that recognize the antigen and induces apoptosis of the impacted cells, preventing response to subsequent antigen challenge (110, 113). Examples illustrating potential utility include preventing production of inhibitory antibodies to recombinant FVIII needed by hemophilia patients (113), and suppression of the production of IgEs for peanut

allergens (155). Nanoparticles formulated with rheumatoid arthritis–associated antigen citrulline and CD22 ligand can also suppress autoantibody production from memory B cells of rheumatoid arthritis patients (156). With this Siglec platform targeting B cells is effective at inducing tolerance in mice naive to the antigen; it is minimally effective for inducing tolerance in antigen-sensitized animals with memory B and T cell responses. In this regard, however, combining alternative approaches for inducing tolerance offer promise for the future (153, 157).

Siglecs have also been implicated in T cell–mediated tolerance (5, 158). The Van Kooyk group has found that dendritic cells or whole animals primed with sialylated antigens can induce T regulatory cells (Tregs) that reduce expression of inflammatory cytokines and suppress activation and proliferation of $CD4^+$ T cells. In mice this effect was mediated by Siglec-E, since antigen uptake by dendritic cells and suppressed production of INF-γ was reduced in mice deficient in Siglec-E (5, 158).

Asthma and Eosinophilia

Eosinophil dysregulation is associated with asthma, eosinophilia, and other allergic diseases (3). Asthma is a chronic condition characterized by mucus-congested airways and increased susceptibility to bronchospasms. Eosinophil recruitment to inflamed airways is a hallmark of asthma (159). Siglec-8 has been identified as a target for eosinophil depletion due to its restricted expression and consistent levels of expression on eosinophils of healthy and asthmatic patients (160, 161). Anti-Siglec-8 has been shown to promote eosinophil killing through NK cell–mediated antibody-dependent cellular cytotoxicity (ADCC) (161). As described above, anti-Siglec-8 antibody also induces eosinophil apoptosis independent of ADCC, an activity that is significantly enhanced by the eosinophil survival factor IL-5 (3, 118, 161). These in vitro activities translate to depletion of eosinophils in IL-5-treated humanized mice (161), and human clinical trials with anti-Siglec-8 for treatment of eosinophil disorders are being conducted by Allakos (162). The recent availability of Siglec-8 knock-in mice provides an important genetic tool to further define the potential for targeting Siglec-8 in eosinophil-mediated disease (163).

Allergy and Anaphylaxis Mediated by IgE

Allergy is a chronic disease wherein the patient is hyperresponsive to innocuous substances in the environment (164). Allergic symptoms range from mild itching and sneezing to life-threatening anaphylaxis. Mast cells are key effector cells responsible for the pathology of allergy and anaphylaxis. They express the high-affinity IgE receptor (FcεRI) that strongly binds allergen-specific IgEs. Allergen cross-linking of the IgE-FcεRI complex triggers an activating signaling cascade that leads mast cells to release presynthesized bioactive mediators, such as histamine and inflammatory cytokines, that cause allergic symptoms (165, 166).

Human mast cells express several Siglecs, including CD33 and Siglec-5, -6, -7, and -8, and low levels of CD22 (3, 76, 77). Although the natural roles of Siglecs in human mast cell biology are currently unknown (166), antibodies to Siglec-8 induced partial inhibition of mast cell activation/degranulation induced by anti-FcεRI, and showed even more profound inhibition when Siglec-8 was cross-linked to the IgE-FcεRI complex (167). Similarly, while anti-Siglec-7 caused no suppression of mast cell degranulation, cross-linking to the FcεRI receptor with secondary antibodies caused inhibited degranulation (168). These results suggested that while Siglecs may not constitutively suppress FcεRI signaling (**Figure 4*b***), they could be recruited to actively suppress mast cell activation, as had been demonstrated for other murine mast cell inhibitory receptors (e.g., FcγRIIb, CD300a) (169, 170).

This idea was supported in experiments using liposomes bearing an antigen (e.g., trinitrophenol, ovalbumin, peanut allergen) alone, or liposomes copresenting the antigen and a synthetic glycan ligand for human CD33 (CD33L). The presence of the CD33L was found to recruit CD33 to the IgE-FcεRI complex and profoundly reduce antigen-induced mast cell degranulation (76) (**Figure 4*c***). Moreover, in transgenic mice with mast cells expressing human CD33, liposomes copresenting antigen with the CD33L completely suppressed antigen-induced passive cutaneous and passive systemic anaphylaxis and desensitized mice from subsequent antigen challenge by reducing antigen-specific IgE both on the mast cell surface and from the circulation. Suppression of antigen-induced mast cell degranulation required that both antigen and CD33L were on the same particle since addition of a mixture of liposomes with CD33L only and liposomes with antigen only induced degranulation equivalent to liposomes with antigen only. In this context, anti-CD33 antibodies caused no inhibition of allergen-mediated degranulation but prevented suppression caused by CD33L, presumably by blocking CD33L-mediated recruitment to FcεRI. Inhibition induced by CD33L is mediated by CD33 recruitment of SHP-1 since no inhibition is observed in SHP-1-deficient mast cells. It is notable that while wild-type murine mast cells do not express murine Siglecs, they contain signaling pathways compatible with human Siglec-mediated suppression of mast cell signaling. Thus, the development of mouse models with mast cell expression of human CD33 and Siglec-8 will be important for assessing the impact of Siglecs in mast cell–mediated disease (76, 171).

Basophils also express FcεRI. Upon allergen cross-linking of IgE-FcεRI, they release histamine and platelet-activating factor that contribute to allergic symptoms (172). Human basophils express CD33 and Siglec-5, -6, and -7. Although the impact of Siglecs on basophil activation is less studied, FcεRI-induced basophil activation was suppressed by antibody-mediated ligation of Siglec-7 to the IgE-FcεRI complex, but not by anti-Siglec-7 antibody alone, suggesting forced colocalization of Siglec to the IgE-FcεRI is also critical for Siglec to inhibit basophil degranulation (168).

Mast cells and basophils can be activated through other receptors, such as TLRs and complement. They participate in host defense against pathogens and diseases other than allergy (173). The possible roles of Siglecs on these other receptors and the potential to exploit them therapeutically in these contexts remain to be determined. Based on evidence to date, recruitment of inhibitory Siglec to the IgE-FcεRI complex or other activating receptors is one strategy to reduce the risk of anaphylaxis and increase safety of allergen immunotherapy.

Cancer—Siglecs as Targets for Immunotherapy

Siglecs have been targets for immunotherapy of hemopoietic cancers for over 40 years based on their selective expression and their ability to transport toxic cargo into the cell by endocytosis (56, 100) (**Figure 4*d***). Indeed, gemtuzumab ozogamicin (GO) (Pfizer), an anti-CD33 immunotoxin conjugated to a derivative of DNA-damaging calicheamicin was the first immunotoxin approved by the FDA (174). GO was first approved to treat acute myeloid leukemia (AML) in the United States in 2000 and was withdrawn due to toxicity and lack of efficacy in 2010. With altered dosing regimens GO was shown to be effective and regained approval for treatment of AML in 2017 (175). There are now numerous clinical trials investigating the utility of anti-CD22 and anti-CD33 in lymphoid and myeloid leukemias/lymphomas (176, 177). As an alternative approach to antibody-based targeting of B cell lymphoma, liposomal docorubicin displaying synthetic ligand CD22 was found to effectively deplete human B cell lymphoma in a mouse model. More recently, chimeric antigen receptor T cell (CART) therapy approaches targeting CD22 or CD33 have shown some promise (178, 179).

Cancer—Targeting Siglec Checkpoints

A major thrust in cancer therapy is targeting immune checkpoints that prevent the immune system from eliciting a robust response against the tumor (180). In particular, cancer cells are known to express ligands for inhibitory receptors on their surfaces. Upon contact with the immune cell (e.g., NK cell or CD8 T cell), inhibitory receptors are recruited to suppress an immune response (180–182) (**Figure 4*e***). The classic example is the inhibitory receptor PD-1 (programmed cell death 1) expressed by activated T cells that normally protect the host from autoimmunity. In the cancer microenvironment the ligand for PD-1 (PD-L1) is upregulated on cancer cells, leading to suppression of antitumor cytotoxic T cells by recruitment of PD-1 to the immunological synapse (181, 183, 184) (**Figure 4*e***). Antibodies blocking either PD-1 or PD-L1 prevent recruitment of PD-1 to the immunological synapse, restoring the ability of cytotoxic T cells to attack the tumor (**Figure 4*f***). There are currently nine approved antibody-based blockbuster drugs that bind to inhibitory receptors PD-1 or CTLA-4 or their ligands (180). While they have remarkable efficacy in some patient subsets, they show little benefit in other patients, leaving major unmet medical need for more effective and/or complementary cancer therapies (180).

Siglecs are also expressed on tumor-infiltrating T cells, NK cells, dendritic cells, and macrophages, and they are gaining attention as immune checkpoint targets for development of therapeutics that exploit them to boost an antitumor immune response (6, 7, 9, 185–187). The interest in inhibitory Siglecs in tumor immunology is heightened by the historical observation that hypersialylation of cancer cells is a hallmark for poor prognosis, and it is believed to help tumor cells escape from immune surveillance (158, 188, 189). Since sialic acids are ligands for inhibitory Siglecs, there is a direct analogy to PD-1/PD-L1, where sialic acids on cancer cells are ligands that could recruit Siglecs to suppress immune responses (**Figure 4*e*,*f***).

Siglecs were not initially thought to play an important role in T cell biology since only minor subsets of peripheral naive human T cells were reported to express Siglec-7 and -9 (190–194). Recently, however, inhibitory Siglecs (CD33, Siglec-5, -7, -9, and -10) were found to be upregulated on tumor-infiltrating $CD4^+$ and $CD8^+$ T cells of various cancers, positioning them to contribute to exhaustion of tumor T cell responses (**Figure 4*e***). In mice, naive T cells also express no Siglecs, but transcription of Siglec-F is highly elevated in both $CD8^+$ and $CD4^+$ T cells in a mouse model of T cell exhaustion (195), and Siglec-E is upregulated on tumor-infiltrating T cells in mouse tumor models (196). The potential for regulation of TCR signaling was shown using Siglec-transfected human Jurkat T cell lines, where Siglec-7 and -9 were found to partially colocalize with the TCR-CD3 complex and inhibit TCR-mediated cell activation by a mechanism involving phosphorylation of the Siglec and recruitment of SHP-1 (e.g., **Figure 3*a*** or ***b***) (194). Von Gunten and coworkers found that target cell killing by Siglec-9-expressing tumor-infiltrating $CD8^+$ T cells was enhanced by neuraminidase treatment of the target cells, consistent with destruction of Siglec-9 ligands preventing recruitment to the site of cell contact (197) (**Figure 4*e***). In this in vitro assay, Fab fragments of a Siglec-9-blocking antibody also activate $CD8^+$ T cell cytotoxicity, consistent with blocking recruitment via Siglec-ligands (**Figure 4*f***), but intact anti-Siglec-9 antibodies inhibited cytotoxicity, suggesting induction of a negative signal (**Figure 3*i***) or antibody-mediated relocalization to the activation complex (196, 197) (**Figure 3*d***). Results to date show that Siglec-9 is an attractive target for boosting a T cell antitumor response, but a better understanding of Siglec-9 regulation of signaling is needed.

NK cells participate in the surveillance, identification, and killing of cancer cells and infected cells and are main effector cells for immune targeting of cancer cells through FcγRIII (CD16)-mediated ADCC (198). NK cells also express many inhibitory receptors, including Siglec-7 and -9 that sense self to prevent damage to normal cells and tissues (1, 7, 9). Numerous in vitro studies

support the idea that glycan ligands expressed by cancer cells translocate Siglec-7/-9 to colocalize with activating receptors through which NK cells deplete target cells (1, 7) (**Figure 4*g***). Indeed, enhancing the interaction between Siglecs and target cells by increasing ligands on the target cells with natural ganglioside ligands or synthetic multivalent ligands decreases cell killing (132, 199, 200), and blockade of this interaction with Fab fragments of anti-Siglec-7/9 antibodies enhance NK cell cytotoxicity (e.g., **Figure 3*b***). However, as observed with $CD8^+$ T cells, intact anti-Siglec antibodies inhibit cytotoxicity, suggesting that they induce an inhibitory activity or translocate the Siglecs to the activating receptor in these assays (132) (**Figure 3*d*,*i***). Thus, while there is strong support for the idea that glycan ligands on cancer cells suppress NK cell killing by recruiting inhibitory Siglec-7 and/or -9, the data do not unambiguously support the concept of using anti-Siglec antibodies to enhance NK cell activity in analogy to use of anti-PD-1 (**Figure 4*f***).

In some cancers, neutrophils can be a predominant leukocyte infiltrating cell type that can play various roles in cancer progression and metastasis, including direct killing of cancer cells (201). Human neutrophils highly express Siglec-9, and in target cell killing assays Siglec-9 was enriched at the site of contact between the neutrophil and target cell (202) (**Figure 3*c***). Blocking the *trans* interaction with anti-Siglec-9 reduced recruitment to the site of cell contact and increased target cell cytotoxicity. Similar results were obtained with mouse neutrophils, where Siglec-E is the predominant Siglec. In a study of a mouse model of lung cancer in which cancer cells were injected intravenously, there were more lung nodules in wild-type mice than in Siglec-E-deficient mice, and knock-in of human Siglec-9 restores lung nodule formation to wild-type levels. However, in the in vivo models, the Siglec-E is also expressed on other leukocytes, so the impact of neutrophils was not directly assessed (202).

Macrophages are another tumor-infiltrating cell type that express Siglecs. Tumor-associated macrophages (TAMs) can suppress immune responses indirectly by creating an anti-inflammatory environment through producing inhibitory cytokines, such as IL-10 and transforming growth factor-β (TFG-β), or through direct interactions with cytotoxic T cells to suppress activation through inhibitory receptors like PD-1/PD-L1 (203). Siglec-E is also a dominant Siglec on murine macrophages. In a subcutaneous syngeneic tumor mouse model Siglec-E deficiency results in more aggressive tumor growth than in wild type, contrary to the lung tumor model described above, and knock-in of human Siglec-9 inhibits the growth rate of tumors to wild-type levels (202). It was hypothesized that glycan ligand expressed by cancer cells engages Siglec-E and inhibits macrophage M2 polarization, which is a protumor phenotype (202, 203). Seemingly contradictory results were obtained with human macrophages that express Siglec-9. Sialylated-MUC1 (MUC1-ST) is commonly upregulated on adenocarcinomas and is a ligand for Siglec-9 (204). MUC1-ST induces macrophages to express a TAM surface marker and inhibit $CD8^+$ T cell proliferation (204). More is involved than simple ligation of Siglec-9, since a V-set-binding Siglec-9 antibody blocks the phenotype induced by MUC1-ST, suggesting the involvement of other receptors, or differential localization of Siglec-9 by these two agents (204).

Another function of TAMs is to phagocytose damaged cancer cells, which results in downstream activation of immune responses by the macrophages and other infiltrating leukocytes. As discussed above, in dendritic cells and B and T cells, Siglec-10 binds to the sialo-glycoprotein CD24 in *cis* to negatively regulate DAMP receptors (1). Recently, Weissman and coworkers provided evidence that Siglec-10 on macrophages can interact in *trans* with CD24 expressed on breast cancer cells to prevent phagocytosis of cancer cells that induce protective immune responses to the cancer (205).

Siglec-15 is another Siglec that has been detected on TAMs (30). Unlike Siglec-E and -9, Siglec-15 does not contain cytoplasmic ITIMs but binds the activating adaptor protein, DAP12, through a transmembrane lysine residue (206). Ligation of DAP12 binding protein is well

characterized to induce activating signaling cascade (207) (**Figure 3*j***). Both human and mouse Siglec-15 preferentially bind sialyl-Tn (Neu5Acα2–6GalNAc) (206). A model cell line that overexpresses sialyl-Tn binds to Siglec-15-expressing macrophages and induces TGF-β production through DAP12 and Syk (30). Siglec-15 suppresses antigen-specific cytotoxic T cell expansion and promotes tumor growth in mouse models of tumor (186). Siglec-15 suppresses activation of T cells through multiple mechanisms. In vivo, Sigelc-15-expressing macrophages produce IL-10, which suppresses the expansion of antigen-specific $CD8^+$ T cells. Siglec-15 also directly inhibits antigen-specific T cell expansion independent of macrophages or IL-10 in vitro (186). Siglec-15 antibody treatment reduces tumor growth especially when given in conjunction with anti-PD-1 (186). Siglec-15 may be a new target for checkpoint blockade to improve existing cancer immunotherapies.

While Siglecs are clearly attractive targets for cancer immunotherapy, the use of anti-Siglecs as an alternative to or in synergy with anti-PD-1 has raised concerns about the number of different tumor-infiltrating leukocytes expressing the same Siglec, the redundancy of multiple Siglecs expressed on a single cell type, and the potential for anti-Siglec antibodies to themselves induce an inhibitory response (e.g., **Figure 3*j***). The Bertozzi group has addressed these potential limitations using an alternative strategy to destroy the Siglec ligands on the cancer cell using tumor-specific antibodies engineered to contain a sialidase/neuraminidase (200, 208). Removing sialic acids expressed on the cancer cell would prevent Siglec recruitment to the immunological synapse and allow activation of an immune response against the cancer (**Figure 4*b***) or alternatively prevent ligand-ligation-induced Siglec-mediated inhibitory signal (**Figure 3*i***). This approach was originally tested in the context of the Fc receptor of cytotoxic NK cells engaging a tumor cell coated with the tumor-specific anti-HER2 antibody, an inhibition of NK cell killing by recruitment of Siglec-7/-9 to the immunological synapse (209) (**Figure 4*g***). Conjugation of a sialidase to anti-HER2 was shown to enhance NK cell–mediated ADCC against tumor cells and overcomes anti-HER2-resistant tumors (208). This same concept would in principle apply to cell killing by cytotoxic T cells, or any other activating leukocyte receptor engaging its ligand on a cancer cell.

An alternative strategy to removing Siglec ligands on the cancer cells is the use of a small-molecule inhibitor of sialyltransferase, which would prevent addition of sialic acids to glycoproteins on the rapidly dividing tumor cells. Indeed, Bull et al. (51, 210) have shown that injection of the sialyltransferase inhibitor 3_{ax}F-Neu5Ac directly into tumors in vivo followed by adoptive transfer of OVA-specific $CD8^+$ T cells (OT-I) results in prolonged survival of mice relative to mice with tumors injected with buffer only. Since the OT-I T cells were required for tumoricidal activity, but do not themselves express Siglecs, it was proposed that other Siglec-expressing cells in the tumor microenvironment play a role in the efficacy of the cytotoxic T cells (210).

Neurodegenerative Disease

Alzheimer disease (AD) is a chronic neurodegenerative disease and the leading cause of dementia among the elderly. One hypothesis for the cause of AD is dysregulated deposition of Amyloid beta (Aβ) plaques in the brain (211). Microglia are resident immune cells in the brain, and they are capable of clearing Aβ plaques through phagocytosis. The failure of microglia to phagocytose plaques is hypothesized to contribute dysregulated plaque buildup that eventually leads to AD (212). Restoring the ability of microglia to phagocytose plaque is one of the hypothesized treatments being tested for AD.

Polymorphisms in the human CD33 gene, which is constitutively expressed in microglia, have been strongly associated with AD susceptibility in genome-wide association studies (213–215). The common polymorphism considered the risk genotype ($rs3865444^C$) results in expression of

two gene products that arise from alternative splicing, a major isoform that is the full-length CD33 (CD33M) (**Figure 1**) and a minor truncated isoform (CD33m) that lacks the sialic acid–binding V-set domain (216). A minor protective polymorphism (rs3865444^{A}) results in exclusive expression of the truncated CD33m isoform missing the sialic acid–binding V-set domain (216). Monocytes from patients bearing the common risk genotype have decreased phagocytic activity compared to cells from patients bearing the rare protective genotype (217). Moreover, transfection of full-length but not truncated human CD33 inhibits phagocytosis of a mouse microglia cell line (218).

The full-length CD33 associated with AD risk is hypothesized to inhibit activating receptors involved in microglia phagocytosis, such as TREM2 (219). An interesting observation is that while the full-length CD33 is prominently on the cell surface, the truncated CD33 associated with CD33m is predominantly found in peroxisomes (220). Thus, if full-length CD33 constitutively colocalizes with TREM2 on the cell surface (e.g., **Figure 3*a*,*b***), and CD33m is physically sequestered in peroxisomes, this alone could account for increased phagocytosis. Since CD33m is missing a V-set domain, an attractive hypothesis is that antibodies to the V-set domain or blocking the ligand-binding site of CD33M might result in a protective phenotype. However, CD33 antibodies that bind to the V-set domain and block sialic acid binding (clone WM53) have not yet been observed to enhance microglia phagocytosis (221). Recently, however, macrophages treated with microparticles decorated with high-affinity ligands of CD33 exhibited more robust phagocytosis of Aβ plaques, suggesting the potential for ligand-based targeting to block suppression of microglia uptake of Aβ plaques (19). Clearly a better understanding of microglial cell phagocytosis is needed to determine how targeting human CD33M could improve microglia phagocytosis and reduce AD risk.

To study the impact of CD33 on microglia phagocytosis, mouse CD33-deficient mice have been used in AD models. Mouse CD33 deficiency leads to reduced plaque burden, and this reduction depends on TREM2 (218, 222). However, unlike human CD33, mouse CD33 does not have cytoplasmic ITIMs but has a positively charged amino acid in the transmembrane domain predictive of binding to DAP12 with an activating ITAM (**Figures 1*a*** and **3*j***). Therefore, since signaling is likely to play a role in human CD33 function in microglia, conclusions reached based on changes in mouse CD33 should be made with caution. In this regard, recently developed human CD33 knock-in mice could be a useful genetic tool to study whether human CD33 contributes to AD pathology (76).

In combination with a CRISPR-Cas9 screen of a mouse microglia cell line and comparing microglia RNA-seq from young versus aged mice, mouse CD22 has been found to be upregulated in aged microglia, reducing phagocytosis through a mechanism involving recruitment by α2–6 linked sialic acid ligands and ITIM-mediated recruitment SHP-1 (223). Consistent with this mechanism, deleting CD22 in a CD22 knockout mouse, or pharmacological removal of sialic acid, enhanced phagocytosis in a CD22-dependent manner (223). Moreover, a CD22 antibody enhanced microglia phagocytosis and cognitive performance in mouse models (223). While these results provide additional support, the relevance of Siglecs for regulation of plaque uptake by microglial cells in neurodegenerative disease and a direct link to the role of CD22 in regulation of plaque phagocytosis by human microglial cells are yet to be established.

OUTLOOK

In the last decade there has been enormous progress in understanding the roles of Siglecs in immune cell functions, and the interest in how they serve as immune checkpoints has grown from an appreciation of their relevance to human disease processes. However, even for the best-studied Siglecs such as CD22, much is yet to be learned. As discussed in detail in this review, regulation of

B cell activation through CD22 is highly context dependent, based on its microdomain localization and the presentation of soluble or membrane-bound antigen to the BCR, or ligands for TLRs (1, 25, 79, 116, 144). Now, using what has been learned to date, experiments to study CD22 function in most contexts can be designed rationally.

With the growing interest of other Siglecs as immune checkpoints in human disease processes as diverse as cancer, AD, autoimmune diseases, and allergies, there is a need to better understand the context of their functions in relevant immune cell types. This will facilitate development of approaches to target Siglecs for therapeutic benefit. There are many experimental challenges to such research. One is the selection of in vitro cellular assays that reflect the role of the Siglec in the disease process. Another is to ensure that a targeting agent, e.g., antibody or a ligand-based probe, perturbs Siglecs in vitro in the same way that it would in vivo. Yet another challenge is to select an animal model that reflects the disease in humans. Since mice are commonly used, it is important to note only a few Siglecs have true orthologs between the two species. Moreover, even for clear orthologs such as human and murine CD22, there are significant differences in their specificity for glycan ligands. Thus, while human Siglec knock-in murine models are clearly helpful, the model should be validated to ensure there is not critical mismatch with its ligands.

With reliable assays and models in hand, it will be increasingly important to consider alternative interpretations of the results to define the mechanism of Siglec function. For example, the use of anti-Siglec antibodies that result in inhibition of cell signaling can be interpreted to mean that ligation of the Siglec induces a negative signal that suppresses activation. But as discussed in this review, the same result could mean that the antibodies alter the localization of the Siglec to the site of an activating receptor where endogenous activated kinases initiate phosphorylation of the Siglec. Distinguishing between such alternatives will be critical to designing rational strategies for therapeutic intervention.

As reflected by the rapid progress in the last few years, it is highly likely that the momentum in understanding the roles of Siglecs as checkpoints in immune responses and their roles in human diseases will continue in the foreseeable future.

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