Siglecs, sialic acids and innate immunity

Paul R. Crocker and Ajit Varki

Siglecs are members of the Ig superfamily that bind to sialic acid (Sia) and are mainly expressed by cells of the hematopoietic system. Until three years ago, only four Siglecs were known, namely sialoadhesin, CD22, myelin-associated glycoprotein and CD33. Since then, a further six human CD33-related Siglecs with features of inhibitory receptors have been identified and shown to be expressed by discrete subsets of leukocytes. Recognition of Sia by these Siglecs could play a role in the regulation of the innate immune system.

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The sialic acid (Sia)-binding Ig-like lectins (Siglecs)[1] were discovered through independent studies on a macrophage lectin-like adhesion molecule named sialoadhesin[2] and a B-cell restricted member of the Ig superfamily (IgSF), CD22 (Ref. 3). Both proteins were found to mediate cell–cell interactions *in vitro* through the recognition of sialylated glycoconjugates[4–8]. The cloning of sialoadhesin[9] revealed striking structural similarities to CD22 and led to the demonstration that two other related IgSF proteins – myelin-associated glycoprotein (MAG) and CD33 – which were not previously known to bind to Sia, were also members of the Siglec family[10,11]. Siglecs are I-type (Ig-type) lectins[12] and are characterized by an N-terminal V-set Ig-like domain, which mediates Sia binding[13], followed by varying numbers of C2-set Ig-like domains (Fig. 1). To date, no Siglec has been shown to recognize any cell-surface ligand other than Sia, suggesting that interactions with glycans containing this carbohydrate are important in mediating the biological functions of Siglecs. Very recently, another Ig superfamily member, CD83, expressed on mature dendritic cells (DCs), has been shown to mediate sialidase-sensitive interactions with one particular cell type[14]. CD83 does not contain the typical V-set and C2-set Ig-like domains characteristic of the known Siglecs. Further analyses will be required to determine whether CD83 should be classified as a Siglec or as an I-type lectin with some Sia binding properties.

One striking feature of CD33 and the six recently described CD33-related human Siglecs (Fig. 1) is their differential expression pattern within the hematopoietic system (Fig. 2). This fact, taken together with the presence of two conserved motifs similar to immunoreceptor tyrosine-based inhibition motifs (ITIMs) in their cytoplasmic tails, raises the possibility that CD33-related Siglecs are involved in regulating cellular activation within the immune system. This article reviews the ways in which Sia recognition by CD33-related Siglecs might contribute to immune functions.

New CD33-related Siglecs in the innate immune system

Human (h) Siglecs-5, -7, -8 and -10 were identified in expressed sequence tag (EST) databases as being homologous to CD33 and were found to possess the key features required for Sia recognition (Fig. 1)[15–20]. Siglec-6 was isolated in a screen for leptin-binding proteins[21], and Siglec-7 was also identified as a natural killer (NK)-cell inhibitory receptor in a redirected killing assay and was designated p75/AIRM-1 (Ref. 22). Siglec-9, a close homolog of Siglec-7, was identified as a Siglec-like cDNA[23,24] and also as a Siglec-like gene[25]. Most recently, a novel mouse (m) Siglec with three Ig-like domains and two ITIM-like motifs, similar to hSiglecs-7, -8 and -9, was identified by one group as a CD33-like EST sequence (named MIS)[26] and by another group as a binding partner for the Src homology 2 domain-containing protein tyrosine phosphatase 1 (SHP-1) in a yeast two-hybrid screen (named mSiglec-E)[27].

The hCD33-related Siglecs contain between two and five Ig-like domains and share a high degree of sequence similarity (Fig. 1). The genes encoding them are clustered on human chromosome 19q13.3–13.4 and appear to have evolved by repeated duplications. Until recently, the only published data on a mCD33-related Siglec concerned a CD33-like protein with two Ig domains but lacking ITIM-like motifs[28]. In phylogenetic analyses, this molecule did not form an exclusive clade with hCD33, instead being loosely associated with several hCD33-related Siglecs[23]. The eventual classification of CD33-related Siglec orthologs in mice will depend on the detailed characterization of their expression patterns, binding specificities and functions.

Monoclonal antibodies (mAbs) recognizing each of the recently discovered human Siglecs have allowed a detailed analysis of their expression patterns on blood leukocytes (Fig. 2). Some are expressed broadly; for example, Siglec-9 is found on neutrophils, monocytes, a substantial fraction of NK cells and B cells, and a minor subset of $CD8^+$ T cells[24]. Others have a much more restricted distribution – notably Siglec-8 – which is expressed on circulating eosinophils[17,18] and at very low levels on basophils[18]. Several Siglecs can be present on the same cell type [e.g. monocytes express CD33 and Siglecs-5, -7, -9 and -10 (Refs 15,16,20,23,24,29)], suggesting some degree of functional redundancy at the cellular level. However, each CD33-related Siglec exhibits a very specific expression pattern among hematopoietic cells, which

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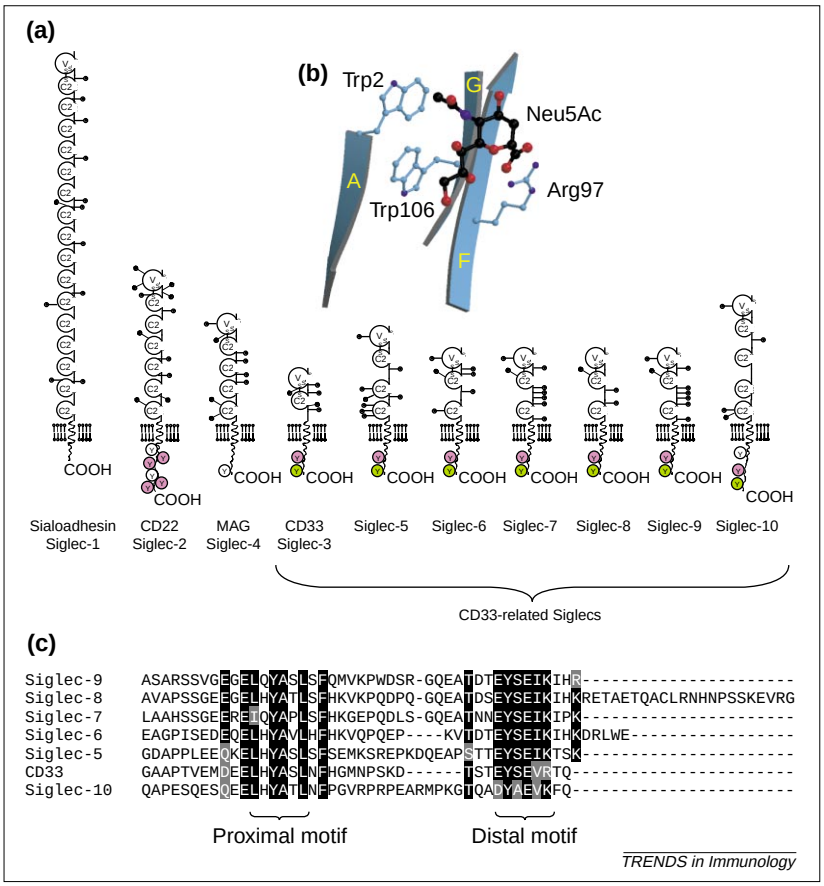

Fig. 1. The currently known human Ig-like lectins that bind to sialic acid (Siglecs). (a) Siglecs are type-I membrane proteins with an extracellular region containing a homologous V-set Ig-like domain and a varying number of C2-set Ig-like domains at the N-terminus. The cytoplasmic tails of all Siglecs apart from sialoadhesin contain tyrosine residues (y) within potential signaling motifs. Those motifs that fit the consensus sequence for an immunoreceptor tyrosine-based inhibition motif (ITIM) are shown in pink. The membrane-distal tyrosine-based motifs that are highly conserved in CD33-related Siglecs are shown in green. The CD33-related Siglecs form a distinct subset of the Siglec family, sharing ≈50–80% sequence identity within the extracellular region. Several Siglecs undergo alternative splicing, but only the known full-length forms are illustrated. Potential N-linked glycans are indicated in ball-and-stick form. (b) Positions of key residues in sialoadhesin that bind the N-acetylneuraminic acid (Neu5Ac) portion of 3′ sialyllactose, as revealed in a ligand-bound crystal structure of the sialoadhesin N-terminal domain[13]. An essential arginine (Arg97) on the F strand (conserved in the other Siglecs) forms a salt bridge with the carboxylate of sialic acid (Neu5Ac) and two tryptophans (Trp2 and Trp106) on the A and G strands form hydrophobic contacts with the N-acetyl and glycerol side groups of Neu5Ac respectively[13]. (c) Alignment of the C-terminal portions of the cytoplasmic tails of CD33-related Siglecs reveals two conserved tyrosine-containing motifs. Residues that are identical are boxed in black and residues that are conserved are boxed in gray. The membrane-proximal motif conforms to the consensus ITIM sequence [(Ile/Val/Leu/Ser)-Xaa-Tyr-Xaa-Xaa-(Leu/Val)], whereas the distal motif does not.

is indicative of specific functions. Limited information is currently available on the expression of CD33-related Siglecs on nonhematopoietic cells. However, Siglec-6 is expressed prominently by cyto- and syncytio-trophoblasts of the placenta, in addition to B cells[21].

CD33-related Siglecs as inhibitory receptors

The importance of balancing positive and negative signals within the immune system is an emerging topic of discussion[30]. When cellular activation is triggered by receptors with immunoreceptor tyrosine-based activation motifs (ITAMs), counteracting inhibitory signals are delivered through receptors bearing ITIMs. Following phosphorylation by Src-family kinases, ITIMs recruit phosphatases, either Src homology 2 domain-containing inositol polyphosphate 5′ phosphatase (SHIP), or more commonly the Src homology 2 domain-containing protein tyrosine phosphatases SHP-1 and SHP-2. These phosphatases inhibit signaling pathways by distinct mechanisms, resulting in raised activation thresholds. The molecular events involved in these processes have been particularly well described for FcγRIIb, a B-cell inhibitory receptor, and the KIRs expressed by NK cells[30].

The presence of two conserved ITIM-like motifs in the cytoplasmic regions of CD33-related Siglecs and the differential expression of these proteins on leukocytes suggests a role in regulating cellular activation. Siglec-8, which originally was thought to lack ITIM-like motifs[17,18], has now been found to exist in alternatively spliced forms containing ITIM-like sequences[20,31]. The membrane-proximal motif of all CD33-related Siglecs conforms to the consensus ITIM [(Ile/Val/Leu/Ser)-Xaa-Tyr-Xaa-Xaa-(Leu/Val)], whereas the membrane-distal motif does not (Fig. 1c). Following the treatment of cells with pervanadate to inhibit tyrosine phosphatases, hCD33, Siglec-7 and mSiglec-E become tyrosine phosphorylated[22,26,27,32–34]. Under these conditions, Siglec-7 recruited only SHP-1, whereas CD33 and mSiglec-E bound to both SHP-1 and SHP-2. Mutation of the tyrosine residue in the distal motif of mSiglec-E did not significantly affect recruitment of SHP-1 and SHP-2, whereas an equivalent mutation in the proximal motif had a major effect on recruitment[26,27,32,33]. The much weaker binding of the distal motif to SHP-1 and SHP-2, as compared with the proximal motif, reflects its departure from the consensus ITIM sequence[27,32]. However, the distal motif is highly conserved in all CD33-related Siglecs, suggesting that it might be important for interacting with other regulatory molecules. Notably, for some CD33-related Siglecs, the distal motif is similar to a motif [(Thr-Ile-Tyr-Xaa-Xaa-(Val/Ile)] in signaling lymphocyte activation molecule (SLAM) and SLAM-related proteins that bind to SLAM-associated protein (SAP), a T-cell-specific molecule that inhibits SHP-2 recruitment to SLAM (Refs 35,36).

Functional evidence that Siglecs can mediate inhibitory signals has been obtained using mAbs to co-crosslink CD33 (Refs 33,34) or mSiglec-E (Ref. 26) with an activating human receptor, FcγR1. This resulted in reduced Ca^{2+} influx compared with crosslinking FcγRI alone. Similarly, Siglec-7 was identified as an inhibitory NK-cell receptor in a redirected killing assay in which anti-Siglec-7 mAb was used to cluster Siglec-7 at the interface between NK cell and target cell[22]. However, the physiological relevance of these interesting findings is unclear because mAbs rather than natural ligands were used to cluster the Siglecs. In other functional studies, the addition of intact anti-CD33 or

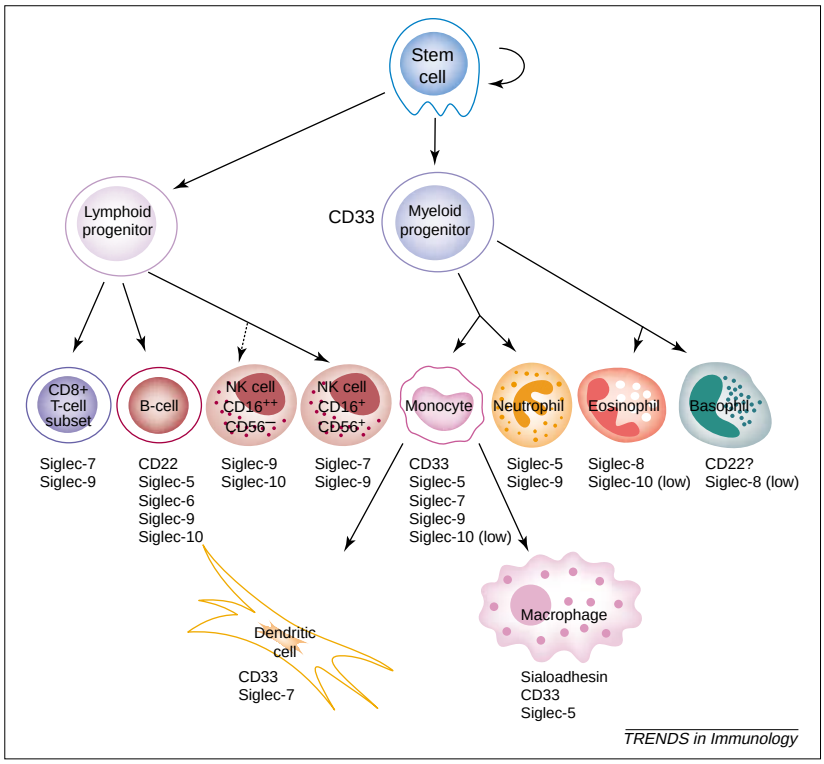

Fig. 2. The expression pattern of human sialic acid-binding Ig-like lectins (Siglecs) within the hematopoietic system. Apart from CD33 and CD22, little is known about the expression patterns of Siglecs on stem cells and progenitors. To date, CD33 and Siglec-7 are the only CD33-related Siglecs reported to be expressed on monocyte-derived dendritic cells. Sialoadhesin, CD33 and Siglec-5 are expressed by subsets of tissue macrophages, but the possible expression of other CD33-related Siglecs on macrophages is unknown. Abbreviation: NK, natural killer.

anti-Siglec-7 mAbs to hematopoietic cell cultures led to reduced cell growth[29] and prevented the development of DCs (Ref. 37). However, it is not clear whether the intact Abs used in these studies also interacted with Ig Fc receptors on myeloid cells.

Table 1. Common sialic acid-bearing structures and their recognition by Siglecs[a]

Oligosaccharide[b]	Relative recognition by Siglec:									
	-1	-2	-3	-4	-5	-6	-7	-8	-9	-10
♦α6●β4■β-R	+	++	++	–	+	–	++	++	++	++
♦α3●β4■β-R	++	–	+	+	+	–	+	++	++	++
♦α3●β3■β-R	++	–	+	+	?	?	?	?	?	?
♦α3●β3□β/α-R	++	–	+	+	?	?	?	?	?	?
♦α3●β3□β/α-R α6 ♦	++	?	+	++	?	?	?	?	?	?
□β/α-R α6 ♦	+	+	+	–	+	+	+/–	?	+	?
♦α3●β4■β-R α3 △	–	–	–	–	–	–	–	?	++	?

[a]Abbreviations: Siglec, sialic acid-binding Ig-like lectin; ♦, Sia; ●, Gal; ■, GlcNAc; □, GalNAc; △, Fuc; ++, robust binding; +, detectable binding; –, no detectable binding; ?, not studied or data not clear.

[b]The effects of sialic acid modifications such as hydroxylation and O-acetylation on Siglec recognition are not considered here. Depending upon the glycan structure, R could be an underlying N-linked glycan, an O-linked glycan or a glycolipid. Glycan chains are designated according to the schema used in *Essentials of Glycobiology*[40].

This could have given rise to 'confused signals' not necessarily relevant to Siglec functions. Clearly, alternative experimental approaches are required to understand the functions of CD33-related Siglecs.

Sialic acid recognition by CD33-related Siglecs and *cis*-interactions with endogenous carbohydrates

Sia is a generic term for a family of nine-carbon sugars that are derivatives of neuraminic acid (Neu) or keto-deoxynonulosonic acid (KDN). Several different types of Sia exist in mammals, and Sia can occur in different glycosidic linkages, typically at the exposed, nonreducing ends of oligosaccharide chains attached to a wide variety of proteins and lipids. Thus, in addition to their roles in masking subterminal sugars and preventing cell–cell interactions through nonspecific charge-repulsion effects, Sia residues are also well suited as ligands for mediating selective cell–cell interactions[38–40].

With the exception of Siglec-6, which shows restricted specificity for the sialyl Tn antigen (Neu5Ac2-6GalNAc-R, where R is usually serine or threonine)[21], all of the known Siglecs recognize forms and linkages of Sia that are commonly found at the cell surface and in the extracellular milieu (Table 1). One consequence of this is that the Siglec binding site can become masked by *cis*-interactions with sialylated ligands on the same plasma membrane (Fig. 3b). In a recent study using multivalent glycoprobes, unmasked forms of Siglecs on human blood leukocytes could not be detected without prior bacterial sialidase treatment to remove the *cis*-inhibitory Sia (Ref. 41). Unmasking also occurred following cellular activation of neutrophils and peripheral blood mononuclear cells by phorbol ester plus ionomycin treatment. These observations are similar to those made previously for CD22, which was shown to be masked on most resting B cells[42,43] but becomes unmasked on cellular activation[42]. Taken together, these data indicate that most Siglecs exist on resting cells in a masked form, but that unmasking might occur during cell activation. Sialoadhesin is an exception to this general theme as it is able to mediate binding to other cells when naturally expressed on stromal tissue macrophages[2,5] (Fig. 3a).

cis-interactions with Sia in the same plasma membrane could potentially regulate Siglec functions in a variety of different ways: (1) by preventing cell–cell interactions until they are required; (2) by facilitating specific interactions with other proteins on the same cell surface; or conversely, (3) by preventing specific protein–protein interactions through the nonspecific engagement of the Siglecs with other sialylated molecules. Regardless of which of these mechanisms is operative in a given cell type, the necessary 'unmasking' of the Siglec could potentially be mediated by a cell-surface sialidase, by

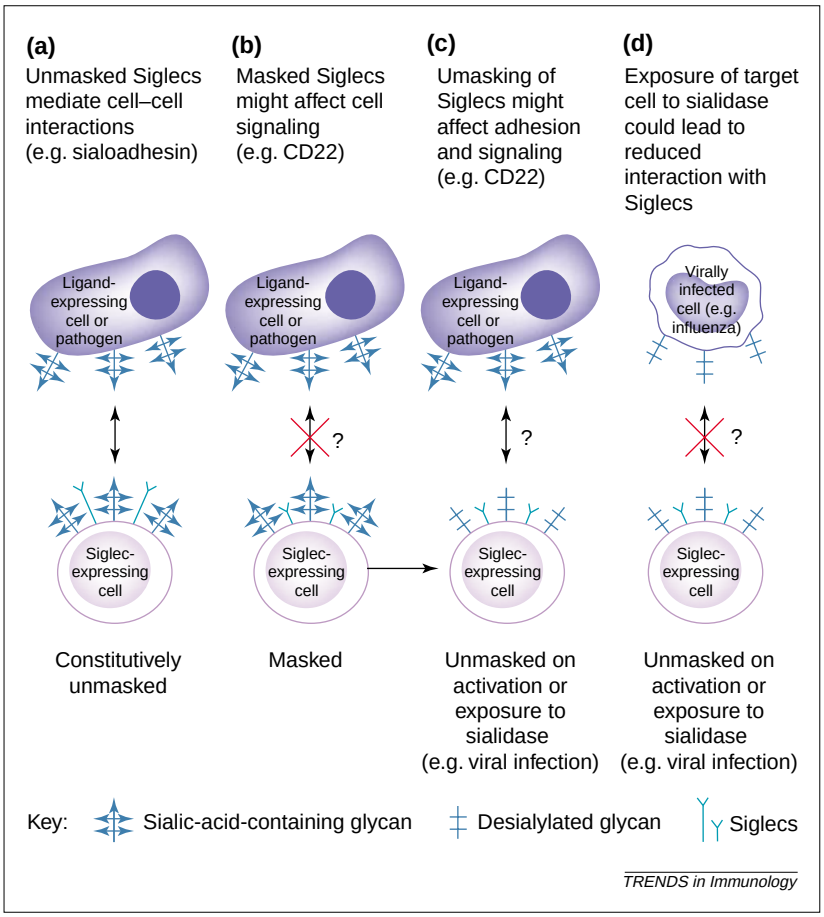

Fig. 3. The interaction of sialic acid (Sia)-binding Ig-like lectins (Siglecs) with cells or sialylated pathogens might be affected by *cis*-interactions with sialylated glycoconjugates. (a) Some Siglecs such as sialoadhesin appear to be unmasked and able to mediate cell–cell interactions constitutively, whereas (b) the majority of Siglecs (including CD33-related Siglecs) appear to be naturally masked owing to *cis*-interactions with adjacent Sia. *cis*-interactions might be important for signaling functions; for example, CD22 is closely associated with the B-cell receptor complex and provides inhibitory signals that raise the threshold of B-cell activation. (c) Unmasking of Siglecs can occur in some cases by cellular activation or by exposure to sialidases. The unmasked Siglec would then be capable of stronger interactions with ligands on other cells or on pathogens. This could result in increased cellular interactions and/or signaling. (d) In another scenario, a virally infected cell expressing a viral sialidase (i.e. neuraminidase) on the cell surface might result in loss of Siglec ligands and reduced interactions with unmasked Siglecs on cells of the innate immune system. In this situation, unmasking of Siglecs could also occur by close cell–cell contact with the virally infected cell.

altered glycosylation or by re-organization of plasma membrane domains (Fig. 3).

Myeloid-cell inhibitory receptors interact with broadly expressed ligands

A large number of ITIM-containing receptors have been discovered in the past few years, many of which are expressed by myeloid cells of the innate immune system[30]. An essential feature of the innate immune system is its ability to distinguish foreign pathogens from self and to make an appropriate orchestrated response that either leads to the direct elimination of the pathogen and/or activation of an adaptive immune response. Bystander damage to host cells is an inherent risk of this process. One way to prevent inappropriate reactivity against self would be to display host-specific ligands that engage inhibitory receptors on effector cells such as granulocytes, NK cells, monocytes and macrophages. Provided these ligands are absent from potential pathogens, host defence functions would not be impeded. For this system to be effective, the ligands would have to be expressed widely, especially in tissues where large numbers of myeloid cells are found and where infections are most likely to occur. For example, MHC class I molecules are displayed on most nucleated cells and serve as ligands for killer inhibitory receptors (KIRs) on NK cells and for the Ig-like transcript 2 (ILT-2) and ILT-4 inhibitory receptors found on myeloid cells[30].

Two further examples highlight the importance of inhibitory receptor–ligand interactions in the regulation of the innate immune system. Human signal-regulatory protein α (SIRP-α), an inhibitory receptor of myeloid and neuronal cells, binds specifically to the widely expressed molecule CD47 (Refs 44–46). When CD47-deficient erythrocytes were injected into normal mice they were rapidly phagocytosed by splenic red pulp macrophages[47]. Thus, CD47 appears to act as a marker of self that prevents inappropriate macrophage phagocytosis by interacting with the inhibitory receptor SIRP-α. Smallpox and vaccinia viruses express CD47 homologs, raising the intriguing possibility that this pathway has been exploited by certain pathogens to enhance survival in their hosts.

Another recently discovered myeloid inhibitory receptor is the OX2 receptor (OX2R), expressed specifically by macrophages and granulocytes[48]. OX2R is a member of the IgSF and has three tyrosine-based motifs. It interacts with OX2, a structurally similar molecule that lacks signaling motifs. In contrast to OX2R, OX2 is expressed widely throughout the body[48]. In OX2-deficient mice, the major phenotypic change was associated with the myeloid cells that express OX2R, resulting in microglia (brain macrophages) with an activated phenotype[49] and increased numbers of macrophages and granulocytes in lymphoid organs[50]. OX2-deficient mice also exhibited a greater susceptibility to and more rapid onset of experimental autoimmune diseases. Interestingly, OX2-like proteins have been identified in several human herpes viruses.

In conclusion, these myeloid-cell inhibitory receptors have broadly expressed self ligands that play a crucial role in modulating functional activities through cell–cell interactions. The acquisition of similar ligands by pathogens might provide them with a survival advantage within the host by inhibiting cellular activation.

Could Sia act as a marker of self in the immune system?

In light of this discussion, it is tempting to speculate that Sia residues could also act as broadly expressed self ligands that contribute to the setting of appropriate activation thresholds by interacting with

Box 1. Sia expression by pathogens and the associated disease

Sia synthesized by the pathogen:
Neisseria meningitidis groups B and C (meningitis and septicaemia),
Haemophilus influenzae (infant meningitis and respiratory infection),
Haemophilus ducreyi (chancroid, a venereal disease),
Escherichia coli groups K1, K92 etc. (neonatal meningitis and septicaemia),
Pasturella hemolytica (bovine bronchitis),
Streptococcus group B (neonatal septicaemia).

Sia transferred from host glycoconjugates by trans-sialidase:
Trypanosoma cruzi (Chagas' disease),
Corynebacterium diphtheriae (diptheria).

Sia captured from host cytidine monophosphate-Sia by surface sialyltransferase:
Neisseria gonorrhoea (gonorrhoea),
Neisseria meningitidis group A (meningitis and septicaemia),

Source of Sia unknown:
Sporotrichium schenkii (fungal skin infection),
Cryptococcus neoformans (meningitis),
Candida albicans (mucosal infections),
Campylobacter jejuni (diarrhoea, Guillian–Barre syndrome).

CD33-related Siglecs on cells of the immune system. This theory could also apply to CD22, a well-characterized inhibitory receptor of B cells that regulates B-cell activation thresholds through *cis*-interactions with the B-cell receptor complex[51]. On the one hand, a Siglec on an effector cell that becomes unmasked upon activation might be re-engaged if there is an adjacent normal host cell with cell-surface Sia (Fig. 3c). On the other hand, if the host cell is infected with a sialidase-producing virus (e.g. influenza), or has been extensively exposed to a secreted bacterial sialidase, its cell surface would be devoid of the Sia that could engage the unmasked Siglec (Fig. 3d). In these scenarios, the re-engagement of the unmasked Siglec (or the absence of re-engagement) could affect Siglec clustering and thus the phosphorylation status of the cytoplasmic ITIM motifs, eventually leading to modulation of the activation state of the effector cell in the appropriate direction. All of these possibilities should be testable in *in vitro* systems.

Do microorganisms that express Sia subvert Siglec function?

Unlike most other common sugars, Sia residues are thought to have appeared relatively late in evolution, being found in deuterostome lineage animals such as starfish[39]. Indeed, recent genomic database searches of *Caenorhabditis elegans* and *Drosophila melanogaster* have failed to uncover obvious sequences encoding known enzymes of the Sia biosynthesis pathway[23]. Despite the rarity of Sia in lower organisms, it is striking that many known pathogens have independently evolved the capacity to synthesize Sia or capture it from their hosts (Box 1). In several cases, expression of Sia has been shown to be essential for pathogenicity and survival within the host. The presence of Sia on these pathogens is likely to be important for host mimicry, prevention of complement activation, attenuation of Ab production and nonspecific charge-repulsion effects. An additional interesting possibility is that Sia residues on pathogens interact with inhibitory CD33-related Siglecs. This could result in reduced activation responses and increased pathogen survival within the host. This might seem an attractive hypothesis, but many unanswered questions remain. For example, can resting leukocytes with masked Siglecs engage Sia on pathogens, or is activation and unmasking required first? If masked Siglecs can interact with Sia on pathogens, can this lead to a downstream host–parasite interaction that is beneficial to the parasite?

Conclusion

Although a great deal is known about the molecular basis for Sia recognition by Siglecs, very little is known of its functional significance. The discovery of several CD33-related Siglecs expressed on cells of the innate immune system is an important addition to the debate. The highly conserved ITIM-like motifs in these proteins are strongly suggestive of inhibitory functions, but precisely how Sia recognition contributes to this is not yet known. We hope that the hypotheses presented here will act as a stimulus for further research that will help to elucidate not only the biological roles of these proteins in host immunity but also, the significance of Sia recognition.

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