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Minireview

# CD33-related sialic-acid-binding immunoglobulin-like lectins in health and disease

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**Abstract**—Sialic-acid-binding immunoglobulin-like lectins (Siglecs) are members of the Ig superfamily that bind sialic acids in different linkages in a wide variety of glycoconjugates. These membrane receptors are expressed in a highly specific manner, predominantly within the haematopoietic system. The CD33-related Siglecs represent a distinct subgroup that is undergoing rapid evolution. The structural features of CD33-related Siglecs and the frequent presence of conserved cytoplasmic signalling motifs point to roles in regulating leukocyte functions that are important during inflammatory and immune responses. In this review, we summarise ligand binding preferences and describe recent progress in elucidating the functional roles of CD33-related Siglecs in the immune system. We also discuss the potential for targeting novel therapeutics against these surface receptors.


*Keywords:* Immunoglobulin superfamily; Inhibitory receptors; Innate immunity; Siglecs; Sialic acid; CD33

**Contents**



## 1. Introduction

Sialic-acid-binding immunoglobulin-like lectins (Siglecs) are type I transmembrane proteins belonging to the immunoglobulin superfamily.[1] They bind sialic acids (Sias), which are commonly found in the terminal region of cell surface oligosaccharides. The Siglec family can be grouped into two subsets based upon evolutionary conservation and sequence similarity. Four Siglecs are conserved in mammalian species: sialoadhesin (Siglec-1, Sn), CD22 (Siglec-2), myelin-associated glycoprotein (MAG, Siglec-4) and Siglec-15.[2] Members of the second group is termed the CD33-related (CD33-r) Siglecs, which exhibit a high degree of sequence similarity with CD33 in the extracellular and intracellular regions. At present, nine CD33-r Siglecs are known in humans (CD33, Siglecs-5 to -11 and Siglec-14) and five in mice

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(mCD33, Siglec-E, Siglec-F, Siglec-G and Siglec-H). However, orthologues are difficult to define due to the high sequence similarity amongst the subgroup and the fact that human CD33-r Siglecs are evolving rapidly. Some CD33-r Siglecs have evolved as hybrids of pre-existing genes by gene conversion.[3] Therefore, sequence composition, gene position, exon structure as well as expression patterns and ligand recognition are all taken into account when defining orthologues or functional equivalents. Hence, a different nomenclature system exists in humans and mice.

The CD33-r Siglecs consist of a Sia binding N-terminal V-set immunoglobulin (Ig) domain and a variable number of C2-set Ig domains connected via a transmembrane domain to a cytoplasmic region usually containing one or more immunoreceptor tyrosine-based inhibitory motifs (ITIMs) or ITIM-like motifs. Ligation of ITIM-bearing CD33-r Siglecs induces rapid (1–3 min) phosphorylation of the tyrosine motifs and recruitment of signalling molecules, src homology region 2 domain-containing phosphatase (SHP)-1 and -2 tyrosine phosphatases and the suppressor of cytokine signalling-3 (SOCS-3).[4–7] Exceptions are human Siglec-14 and mouse CD33 and Siglec-H, which lack ITIMs. Indeed, Siglec-14 and Siglec-H have been shown to interact via their charged transmembrane residues with adaptor proteins like DNAX activating protein of 12 kDa (DAP12 or KARAP), an ITAM-containing adaptor that triggers both activating and inhibitory signalling.[8–10] The same tyrosine-based motifs are important in endocytic function of CD33-r Siglecs.[11–13]

The CD33-r Siglecs are expressed differentially on leukocyte subsets and thus are thought to be involved in regulation of leukocyte functions during inflammatory and immune responses.[14,15] Some such Siglecs are highly restricted in their expression pattern; for example, Siglec-8, which is found predominantly on circulating eosinophils[16,17] and at very low levels on basophils,[17] and Siglec-H, which is an excellent marker of rodent plasmacytoid dendritic cells.[9,18] In contrast, other Siglecs demonstrate broad expression patterns; for example, Siglec-9 and Siglec-E are found on monocytes, neutrophils, dendritic cells and on the subsets of NK cells and B cells.[15,19] Several Siglecs can be present on the same cell type. For example, human monocytes have been shown to express, CD33 and Siglecs-5, -7, -9 and -10, suggesting functional redundancy amongst these receptors.[19–24] However, analysis of fine sugar binding specificity has shown that each Siglec exhibits a unique sugar binding pattern (Table 1),[25,26] arguing that even when several Siglecs are expressed simultaneously, each may perform a unique function in fine-tuning cellular activation and/or endocytosis.

A comprehensive review of Siglecs in the immune system including CD22 and sialoadhesin has been published recently.[1] The focus of this review is to describe recent progress in elucidating the functional roles of the CD33-r Siglecs in the immune system and to discuss these Siglecs as potential targets for therapeutics.

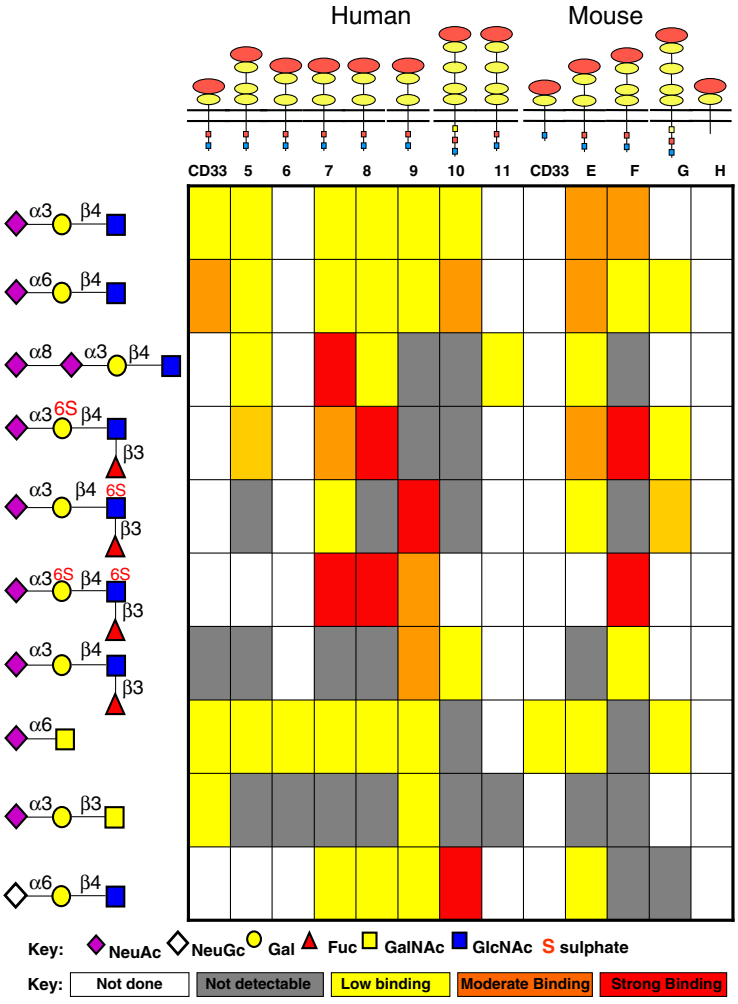
## 2. Linking sugar recognition to function

Sialic acid (Sia) refers to a family of nine-carbon sugars that are mostly derived from *N*-acetylneuraminic acid (Neu5Ac). Several different types of Sia exist in Nature, although mammals mainly express Neu5Ac, *N*-glycolylneuraminic acid (Neu5Gc) and 5,(7)9-*N*,*O*-diacetylneuraminic acid (Neu5,(7)9Ac2). Humans lack Neu5Gc owing to a mutation in the *CMAH* (cytidine monophosphate-*N*-acetylneuraminic acid hydroxylase) gene, which encodes the enzyme required for the conversion of Neu5Ac to Neu5Gc. Sia can occur in different glycosidic linkages, typically at the exposed, non-reducing ends of oligosaccharide chains attached to a wide variety of proteins and lipids. They are transferred using α2–3, α2–6 or α2–8 linkages to subterminal sugars by a family of about 20 sialyltransferases. Thus, the primary regulation of Siglec ligand formation is likely to be dictated by the differential expression of various sialyltransferases in different cell types.

The CD33-r Siglecs have a unique specificity for sialylated ligands indicative of specific functions. Siglec binding to glycan ligands has been shown to be greatly influenced by the type of Sia, its linkage to the subterminal sugar, the structure of the underlying glycan and other modifications such as sulfation. Laboratories tend to vary in their use of glycan derivatives and assay formats for analysing Siglec–glycan interactions. Apart from real-time interactions measured by surface plasmon resonance, Siglecs need to be multimerised to see stable binding due to their very low affinity for sialoglycoconjugates (dissociation constants are typically 100–1000 rmuM). Whilst results are somewhat variable depending on the assay format and Siglec preparation, there is nevertheless a general consensus regarding specific structures recognised by some of the CD33-r Siglecs, namely, Siglecs-7, -8, -9 and mSiglec-F.[15,26–29] Table 1 compares the sialoside specificities derived from competitive binding experiments and from glycan array analysis for the CD33-r Siglecs against selected sialoside sequences found in mammalian glycoproteins and glycolipids.

Siglec-8 and Siglec-9 have differential specificity for sulfated forms of $SLe^x$, with the position of the sulfate on either the Gal or GlcNAc being an important determinant of specificity.[26,27,29,30] A doubly sulfated form of $SLe^x$ is recognised broadly by several Siglecs tested (Table 1), and if both sulfate groups are removed, only Siglec-9 shows a clear preference (Table 1). Analyses of the glycan binding specificities of Siglec-7 and Siglec-11 demonstrate preferences for sialosides with the Neu5A-

**Table 1.** Structure and glycan binding properties of the CD33-related Siglecs




The structures of human and murine CD33-related Siglecs are shown, as well as glycan binding specificities. Sialic-acid-binding immunoglobulin-like lectins (Siglecs) are type 1 membrane proteins containing an amino-terminal V-set immunoglobulin domain that mediates sialic acid recognition and varying numbers of C2-set immunoglobulin domains. The CD33-related Siglecs differ in composition between species, share high sequence similarity in their extracellular regions and frequently contain conserved tyrosine-based signalling motifs in their intracellular domains (key for Siglec structure is shown in Fig. 1).

The table shows a comparison of CD33-related Siglecs binding to sialylated glycans obtained from published competitive binding experiments and from glycan array analysis (also see the Consortium for Functional Glycomics website: http://www.functionalglycomics.org). The table shows that each Siglec has a distinct preference for specific types of sialic acid and also for specific types of linkage to subterminal sugars. Relative strengths of binding are depicted by a colour map where white = not determined; grey = no binding detected; yellow = low binding; orange = moderate binding and red = strong binding.

c(α2–8)Neu5Ac structure.[8,31] Siglec-6 shows restricted specificity for the sialyl Tn antigen (Neu5Ac2-6GalNAc-R, where R is usually serine or threonine).[32]

When Siglecs are expressed on cells, ligand recognition is complicated by the fact that these receptors interact with their sialic-acid-containing ligands both in cis and trans.[33] In this regard, interactions with cis ligands are a common feature of Siglecs, resulting in the masking of Siglec function (discussed in detail in Ref. 1). The future identification of high affinity, selective ligands for the CD33-r Siglecs will provide useful tools for distinguishing unmasked, 'Siglec-competent' subsets of cells from masked subsets. Cells expressing unmasked Siglecs are more likely to interact with ligands expressed on other cells or in the extracellular environment and modulate cellular functions such as proliferation, survival and activation. Thus, Siglec unmasking combined with upregulation of Siglec ligands could be an important mechanism for regulating inflammatory responses as discussed further below.

## 3. Functional role of CD33 related Siglecs—lessons from Siglec-deficient mice

To date, the functions of CD33-r Siglecs have been investigated predominantly in studies using Siglec-transfected cells and/or antibody crosslinking. The results suggest that Siglecs are important for regulating leukocyte functions during inflammatory and immune responses including cell proliferation, differentiation, apoptosis, activation, survival and endocytosis.[1,34–37] The analysis of mice deficient in functional Siglecs will be of great value in identifying the biological roles of Siglecs in vivo. However, the data may be difficult to interpret due to overlapping expression of Siglecs in various blood and immune cell types. No overt phenotype was initially seen in CD33-deficient mice.[38] This may be because, in contrast to human CD33, mouse CD33 lacks the cytosolic ITIM. Moreover, the expression pattern differs in that mouse CD33 is expressed predominantly on neutrophils rather than monocytes in humans.[38] Recently, there have been reports of mice deficient in ITIM-bearing CD33-r Siglecs: Siglec-F[39] and Siglec-G[40] which will be discussed in detail below.

### 3.1. Siglec-F

Siglec-F has four extracellular immunoglobulin-like domains, a transmembrane domain, and cytoplasmic tail, which contains a putative ITIM in addition to a C-terminal tyrosine-based ITIM-like motif.[41] Murine Siglec-F is expressed on mature circulating eosinophils and following allergen exposure, on activated $CD4^+$ T cells during a model of allergen-induced airway inflammation.[15,29,39] It is also a very useful marker for the studies of eosinophil function and turnover.[42,43] Siglec-F shows weak binding to α-2,3 linked Sias, but has a strong preference for 6′-sulfo-sialyl Lewis X, the same ligand that is selectively recognised by Siglec-8 (Table 1).[27,29] Although Siglec-F is not orthologous with Siglec-8, their similarities in expression pattern[16] and ligand preference[29] suggest that these paralogues play equivalent functional roles. Therefore, analysis of Siglec-F-deficient mice is likely to give insights into Siglec-8 functions in humans.

In a model of allergen-induced airway inflammation, expression of both the Siglec-F on eosinophils and its sialic-acid-containing ligands in the inflamed airways was shown to be elevated following an allergen exposure of sensitised mice in comparison to sham controls.[39] Moreover, Siglec-F-deficient mice demonstrated enhanced eosinophilic inflammation possibly due to delayed resolution and diminished apoptosis.[39] Furthermore, in vitro crosslinking of Siglec-F on the surface of eosinophils was shown to induce apoptosis, a phenomenon also observed following antibody-induced crosslinking of Siglec-8 on human eosinophils.[44] Therefore, it has been proposed that Siglec-F expressed on eosinophils might provide a negative feedback loop in regulating allergic responses.

In general, mouse T cells do not express Siglecs although CD22 has been reported on primary mouse T cells.[45] Recently, it has been demonstrated that upon activation, CD4 and CD8 T cells in vitro and CD4 T cells in vivo express Siglec-F.[39] Therefore, it can be hypothesised that Siglec-F expressed on eosinophils and activated T cells in combination with an increased expression of sialyated ligands provide a negative feedback loop in regulating allergic responses in the described model. These data from the Siglec-F-deficient mouse translate to the current knowledge of Siglec-8 in humans and together provide evidence consistent with an inhibitory role of CD33-r Siglecs in controlling leukocyte expansion during inflammation. Thus, novel therapeutics designed to target Siglec-8 may aid the treatment of diseases where eosinophils are a major infiltrating population, for example, allergic asthma and parasitic disease.

### 3.2. Siglec-G

Murine Siglec-G is the orthologue of human Siglec-10 with high sequence identity, similar chromosomal location of their genes and conserved structure of the proteins.[41] Siglec-10 and mouse Siglec-G contain five extracellular Ig domains and an intracellular domain with three conserved tyrosine-based motifs including an ITIM.[41,46] Siglec-10 is expressed on B cells and on subpopulations of eosinophils and monocytes, and its intracellular ITIM binds to SHP-1 and SHP-2.[22,47] Similarly, the Siglec-G gene is also expressed in the B cell lineage of the mouse, including pre B cells and B1a cells of the peritoneal cavity[40] and in eosinophils.[46] Therefore, generation of the Siglec-G-deficient mouse provides an ideal system to study in vivo functions of Siglec-10.

Siglec-G-deficient mice demonstrate a striking, ~10-fold increase in the number of B1a cells. This enhanced expansion begins early in development and, using bone marrow chimaeras, was shown to be an intrinsic property of the Siglec-G-deficient B cells.[40] B1a cells develop early in ontogeny and have unique properties, including 'natural' antibody production and rapid, T cell-independent antibacterial immunoglobulin responses. Siglec-G-deficient mice had higher titres of natural IgM antibodies but not of IgG autoantibodies. Moreover, enhanced calcium signalling was observed in Siglec-G-deficient B1a cells supporting the evidence of Siglec-G-dependent negative regulation in B1a cells. Further studies using the Siglec-G-deficient mouse will prove invaluable in investigating further functions of Siglec-G in vivo which may elude to the functions of Siglec-10 in man.

In conclusion, these recent data demonstrate that mouse models are useful tools for investigating the functional roles of CD33-r Siglecs in vivo. Further studies on

these mice are likely to lead to a better understanding of the biology of human Siglecs and their roles in regulating immune and inflammatory responses. One potential issue with the single knock-out mice may be functional redundancy of Siglecs, as in some cases one cell type may express several different Siglecs. However, knocking out multiple CD33-r Siglecs by intercrossing individual knock-outs is impractical due to their close linkage on chromosome-7 and deletion of the entire cluster of CD33-r Siglecs by gene targeting would be complicated by the presence of interspersed non-Siglec genes.[3]

## 4. Endocytosis and pathogen recognition by CD33-related Siglecs

Antibody ligation of several CD33-r Siglecs has been shown to trigger their endocytosis.[11–13,18] This property could be important in the clearance of sialylated antigens, sialylated pathogens and/or in promoting or inhibiting antigen presentation and in regulating the turnover of Siglecs via lysosomal degradation. It could also be important in regulating their signalling function since the tyrosine motifs of Siglecs are likely to compete with effectors involved in both the signalling and endocytosis pathways. The endocytic capacity of CD33-r Siglecs has also been exploited for therapy as discussed further below.

Although Sias are thought to have appeared relatively late in evolution,[37] many human pathogens have evolved the capacity to express Sia, either by synthesising their own Sia, for example, pathogenic bacteria group B *Streptococcus*, or by transfer of Sia from the host glycoconjugates using trans-sialidases, for example, *Trypanosoma cruzi*. Sia can also be captured from the host CMP-sialic acid by a cell surface sialyltransferase, for example, *Neiserria gonorrhoea*. Thus, pathogens expressing Sia become potential ligands for Siglecs. Indeed, several sialylated pathogens have been shown to bind or be taken up by Siglecs.[48–50] Siglec-5 and Sn have been shown to bind to Neu5Acα2–3Gal expressed on *Neisseria meningitidis* leading to increased pathogen uptake by macrophages.[50] Similarly, Siglec-7 binding to *Campylobacter jejuni*-expressed Neu5Acα2–8Neu5Acα2–3Gal increased pathogen binding to NK cells and monocytes.[48] In addition, several CD33-r Siglecs have been shown to bind to Neu5Acα2–3Gal expressed on the capsular polysaccharide of Group B *Streptococcus*.[49] Therefore, these data provide evidence that the CD33-r Siglecs family can function as receptors for the clearance of pathogens as part of the innate immune response.

Tateno et al. have recently compared and contrasted the mechanisms of endocytosis of CD22 and a CD33-r Siglec, Siglec-F.[12] Ligation of CD22 and Siglec-F-induced endocytosis were both shown to be dependent on the cytoplasmic ITIM and ITIM-like motifs. Endocytosis of CD22 was mediated by a clathrin-dependent mechanism and led to CD22 trafficking to early endosomes and recycling compartments. In contrast, Siglec-F endocytosis targeted the receptor to late endosomal and lysosomal compartments and was mediated by a mechanism that is independent of clathrin and dynamin. It is likely that other CD33-r Siglecs will show similar endocytic properties to Siglec-F since they share highly conserved cytoplasmic tyrosine-based motifs. Even for those CD33-r Siglecs that lack ITIMs, endocytosis is an important function. For example, we have shown that Siglec-H, expressed on plasmacytoid dendritic cell precursors, can rapidly endocytose anti-Siglec-H antibody into endosomal compartments.[18]

## 5. CD33-related Siglecs as therapeutic targets

Acute myeloid leukaemia (AML) is the most common type of acute leukaemia occurring in adults. Approximately 90% of patients have myeloid blast cells expressing the CD33 surface antigen and since CD33 is reported to be absent from haematopoietic stem cells, it provides a selective AML target.[51] Gemtuzumab ozogamicin, a humanised anti-CD33 monoclonal antibody coupled to the antibiotic calicheamicin, has been used as a therapy for relapsed acute myeloid leukaemia. On binding of Gemtuzumab to CD33, the immunotoxin is rapidly internalised,[52] and calicheamicin is released in lysosomes due to their acidic pH. Subsequent binding of calicheamicin to the minor groove of DNA causes double strand breaks and triggers apoptotic cell death[53] (Fig. 1). Since all CD33-r Siglecs tested so far undergo antibody-mediated endocytosis, it is reasonable to speculate that other members of this subgroup could be suitable targets in AML or other haematological disease. Indeed, Siglec-5 has been reported as a marker for myeloid leukaemias[54] and two studies have shown that several other CD33-r Siglecs are expressed in a variable manner on primary cells from AML patients but absent from early progenitors.[11,55] Apart from CD33, Siglec-9 was the most highly expressed of the CD33-r Siglecs on AML cells[11] and can mediate endocytosis of anti-Siglec-9 mAb in an ITIM-dependent manner. These features suggest that Siglec-9 provides not only another useful marker for certain subsets of AML, but also a potential therapeutic target.

The cell specific expression of Siglec-8 on eosinophils may permit selective targeting of these cells in eosinophil-mediated pathologies, for example, allergies, asthma, parasitic disease. Indeed, in vitro crosslinking of antibodies against Siglec-8 have been reported to cause apoptosis in eosinophils.[36] Similarly, antibody crosslinking of Siglec-9 was shown to induce apoptosis of neutrophils.[56] Therefore, in acute and chronic inflammatory conditions where granulocytes induce disease-associated

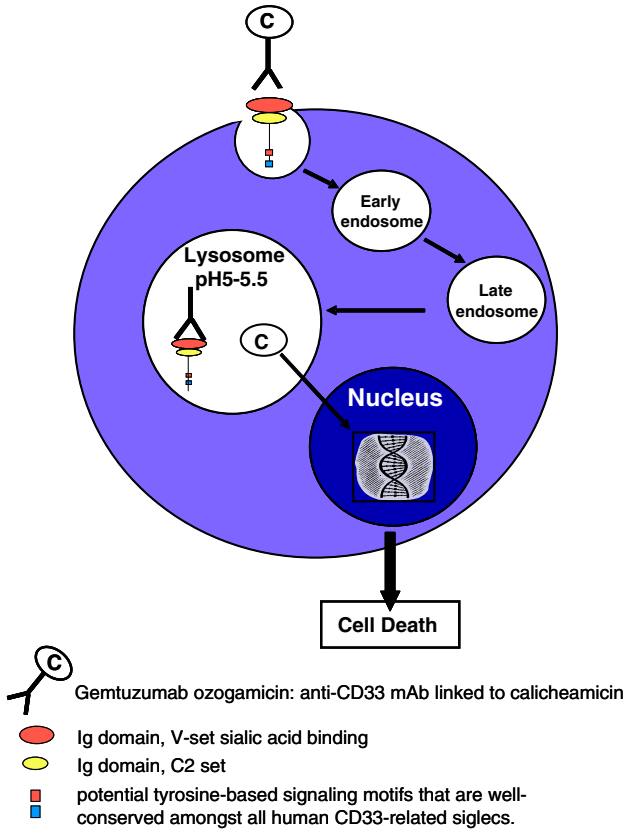



**Figure 1.** Schematic representation of the mechanism of action of Gemtuzumab ozogamicin on a leukaemic cell. Gemtuzumab ozogamicin (GO) is composed of a humanised monoclonal antibody (anti-CD33) coupled to calicheamicin via a bifunctional linker. Typically 2–3 molecules of calicheamicin are bound to anti-CD33. Upon binding to CD33, Gemtuzumab ozogamicin is rapidly internalised via receptor mediated endocytosis and directed via endosomes to lysosomes. Due to the low pH (5–5.5) within the lysosome, calicheamicin is released from the anti-CD33 antibody conjugate and on entry into the nucleus is free to bind double stranded DNA, leading to cell death.

pathology, there is great potential for Siglecs as therapeutics. In addition, it has been demonstrated recently that neutrophil[57] and eosinophil[58] death following administration of IVIg, a plasma protein replacement therapy (IgG) for immune deficient patients, was mediated by naturally occurring anti-Siglec-9 and -8 and autoantibodies present in IVIg. Therefore, anti-Siglec-9 and anti-Siglec-8 autoantibodies present in IVIg may have therapeutic relevance in autoimmune and allergic diseases, respectively.

## 6. Conclusions

CD33-r Siglecs are expressed abundantly on many cells of the immune system and are proving to be important inhibitory receptors for setting thresholds of activation and for regulating cellular proliferation and apoptosis. Further studies using antibody-based approaches in combination with studies of mice deficient in functional Siglecs will aid the understanding of the biological roles of Siglecs. This in turn may lead to novel approaches to the treatment of certain autoimmune and inflammatory conditions.

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