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Commentary

Targeting Siglecs—A novel pharmacological strategy for immuno- and glycotherapy

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ABSTRACT

The immune system must be tightly held in check to avoid bystander tissue damage as well as autoreactivity caused by overwhelming immune reactions. A novel family of immunoregulatory, carbohydrate-binding receptors, the Siglecs (sialic acid binding immunoglobulin-like lectins), has received particular attention in light of their capacity to mediate cell death, anti-proliferative effects and to regulate a variety of cellular activities. Siglec receptors are mainly expressed on leukocytes in a cell type-specific and differentiation-dependent manner. Siglecs might potentially be exploited as targets of novel immune- and glycotherapeutics for cell-directed therapies in autoimmune and allergic diseases, as well as in haematologic malignancies. Here we present novel insights on structural and functional characteristics, expression patterns and evolutionary aspects of Siglecs and their ligands. Pharmacological strategies using Siglec agonistic cross-linking therapeutics, such as monoclonal or engineered antibodies, intravenous immunoglobulin (IVIG), or glycomimetics are discussed. Modulation of immune responses by targeting Siglecs using agonistic or antagonistic therapeutics may have important clinical implications and may pave the way for novel pharmacological avenues for the treatment of autoimmune and allergic diseases or for tumor immunotherapy.

1. Introduction

Since their definition in 1998, Siglecs (sialic acid-binding immunoglobulin-like lectins) have emerged as a novel family of regulatory receptors expressed predominantly on immune and haematopoietic cells [1–4]. Characteristically, each Siglec receptor binds specifically to one or more distinct sialic acid-containing carbohydrates (sialoglycans) [2]. Siglecs have been shown to be involved in a variety of inhibitory processes [2,4,5], such as anti-proliferative effects [6], cell death induction [7,8], suppression of cellular activities [9]. Moreover, they have been implicated in cell–cell interaction processes and endocytosis [10]. Based on their cell type-restricted and differentiation-dependent expression on distinct immune cell types, Siglecs represent attractive targets for cell-directed therapies. As discussed below, to date two Siglec members, CD22 (Siglec-2) and CD33 (Siglec-3), have been successfully targeted by specific monoclonal antibodies in patients with hematologic malignancies and autoimmune diseases, respectively. Proceeding from results of preclinical and clinical studies, it is tempting to speculate that other Siglecs will develop into targets for cell-based treatment strategies for autoimmune, inflammatory and malignant disorders in the near future.

2. Structural characteristics of Siglecs

Siglecs are single pass (type I) transmembrane proteins consisting of an extracellular N-terminal V-set immunoglobulin domain responsible for sialic acid recognition, followed by variable numbers (from 1 to 16) of C2-type Ig repeats, that act as spacers in projecting the ligand-binding site away from the membrane surface (Fig. 1). The V-set domain includes an essential arginine residue, which is absolutely required for sialic acid binding. In this regard, mutations of this residue in human Siglec-12, as well as other Siglecs in different species, resulted in loss of ligand binding ability of these Siglec family members [11]. Intracellularly, most of the Siglecs contain combinations of tyrosine motifs, including one or more membrane-proximal immunoreceptor tyrosine-based inhibitory motif (ITIM) and a membrane-distal ITIM-like motif containing Grb2-binding or Fyn kinase phosphorylation sites [12]. These motifs are involved in inhibitory signal transduction through recruitment of SHP-1, SHP-2 tyrosine phosphatases as well as other scr-homology 2 (SH2)-domain containing effector proteins [13]. As an exception to this common intracellular structure, the newly described Siglec-14, -15 and -16 in humans as well as Siglec-H in rodents lack ITIM or ITIM-like motifs. In contrast, they possess positively charged residues in the transmembrane region, which enable association with intracellular ITAM-bearing adaptor proteins (e.g. DAP12) and subsequent cellular activation [14,15].

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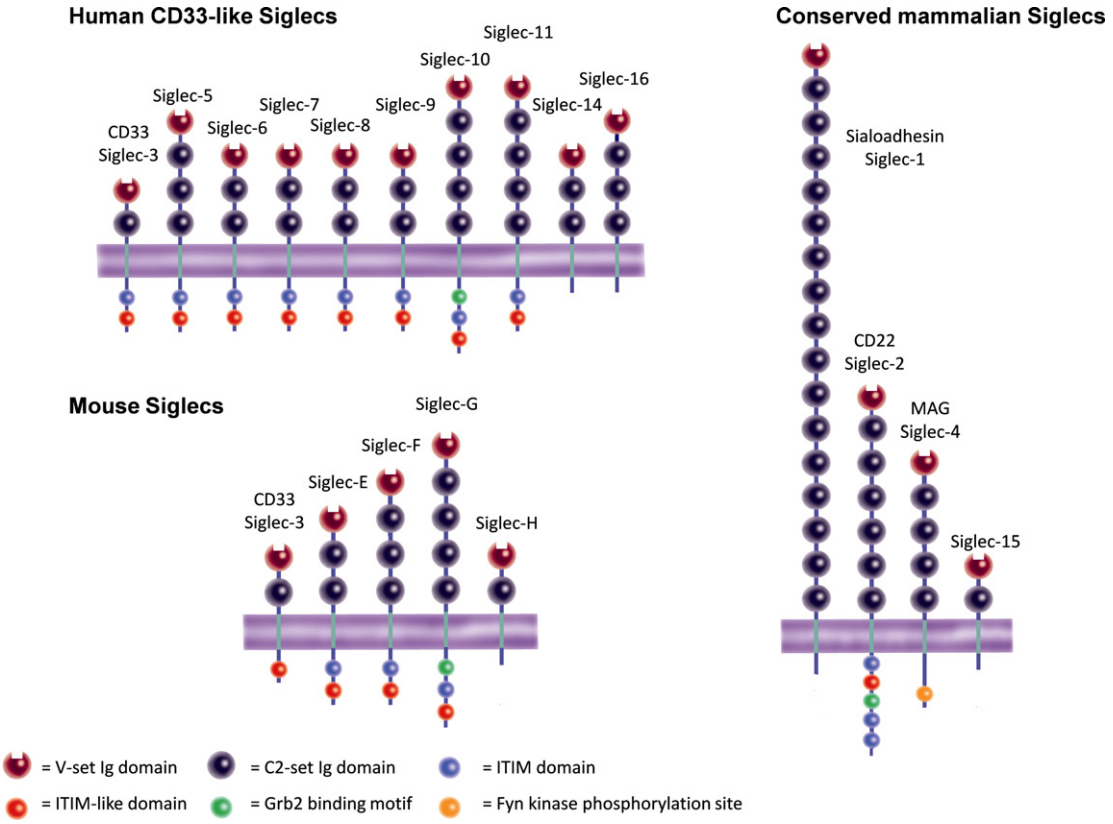

Fig. 1. Nomenclature and structure of Siglecs in humans and mice. The evolutionary conserved Siglec subgroup is shown on the right, the rapid evolving CD33-related Siglec subfamily is depicted on the left (human CD33-related Siglecs on the top, mouse on the bottom). See key for symbols representing extracellular and intracellular domains. Siglec-12 has lost its lectin activity in humans and its name has therefore been changed to Siglec-XII (not shown). *Siglec-13* gene is deleted in humans. Data are compiled from recent original work and reviews [2,4,113].

Siglecs can be broadly categorized into two major subgroups based on both sequence similarity and conservation between mammalian species (Fig. 1). The first, evolutionary conserved subfamily comprises Siglec-1 (also called sialoadhesin or CD169), Siglec-2 (also called CD22), Siglec-4 (also called myelin-associated glycoprotein, MAG) and the recently discovered Siglec-15. These Siglecs show single clear-cut orthologs between all mammalian species examined to date and share only poor sequence homology (25–30%) among each other [2]. The second subfamily, the CD33/Siglec-3-related Siglecs, forms a large cluster on chromosome 19q13.3–q13.4 and 7 in humans and in mice, respectively, and a similar well conserved cluster in all mammals studied until present. CD33-related Siglecs comprise 10 members in humans (Siglec-3, -5, -6, -7, -8, -9, -10, -11, -14, -16), while only 5 in rodents (Siglec-3, -E, -F, -G, -H). These receptors share 50–99% sequence identity [2] with however very poor species homology, probably as a consequence of very rapid evolutionary events (see below). Therefore, it is difficult to define orthologs even between human and rodents, resulting in the current use of different numbering systems between humans and mouse CD33-related Siglecs (in humans they are numbered, in the murine system they are lettered).

3. Cell type-specific and differentiation-dependent expression of human Siglecs

The subgroup of evolutionary conserved members has a restricted expression pattern with Siglec-1 being specifically expressed on macrophages, CD22 (Siglec-2) on B cells and Siglec-4, which is expressed by oligodendrocytes and Schwann cells in the nervous system [16]. In contrast, CD33-related Siglecs display a more divergent expression scheme on cells of the haematopoietic and immune system, in both humans and mice. Fig. 2 illustrates the currently known expression pattern of Siglecs on human haematopoietic cells. Besides Siglec-4, non-haematopoietic expression exhibit Siglec-6, which is expressed on placental trophoblasts [17], Siglec-11, that is present on ovarian stromal fibroblasts [18] and Siglec-XII, that is found on epithelial cells [11]. In this regard, it is striking that with the exception of resting T cells, virtually all cell types in the human and mouse immune system express at least one Siglec family member, with some cells expressing multiple CD33-related Siglecs. It has been postulated that the human-specific loss of Siglec expression on T cells during evolution might contribute to the documented decreased threshold of activation of human T cells and might also help to explain the increased prevalence and severity of T cell mediated diseases in humans (such as AIDS and chronic active hepatitis) as compared to closely related great apes, e.g. chimpanzees [6].

The Siglec expression pattern of a given haematopoietic cell changes during maturation, with acquisition of specific Siglecs at later developmental stages [19]. For instance, immature neutrophils gain Siglec-9 expression not before the myelocyte stage but before CD16 expression [8]. Interestingly, the surface expression and function of several Siglec members (e.g. Siglec-1, -8, -9) have also been shown to be influenced by cytokines, toll-like receptor activation, viral and bacterial infections, and other stimuli, accounting for even further complexity in Siglec biology [4]. The cell type-restricted and differentiation-dependent expression pattern, as well as the specific characteristic ligand recognition profile of each individual Siglec member, lead to cell-specific, unique and non-overlapping functions in defined cell types.

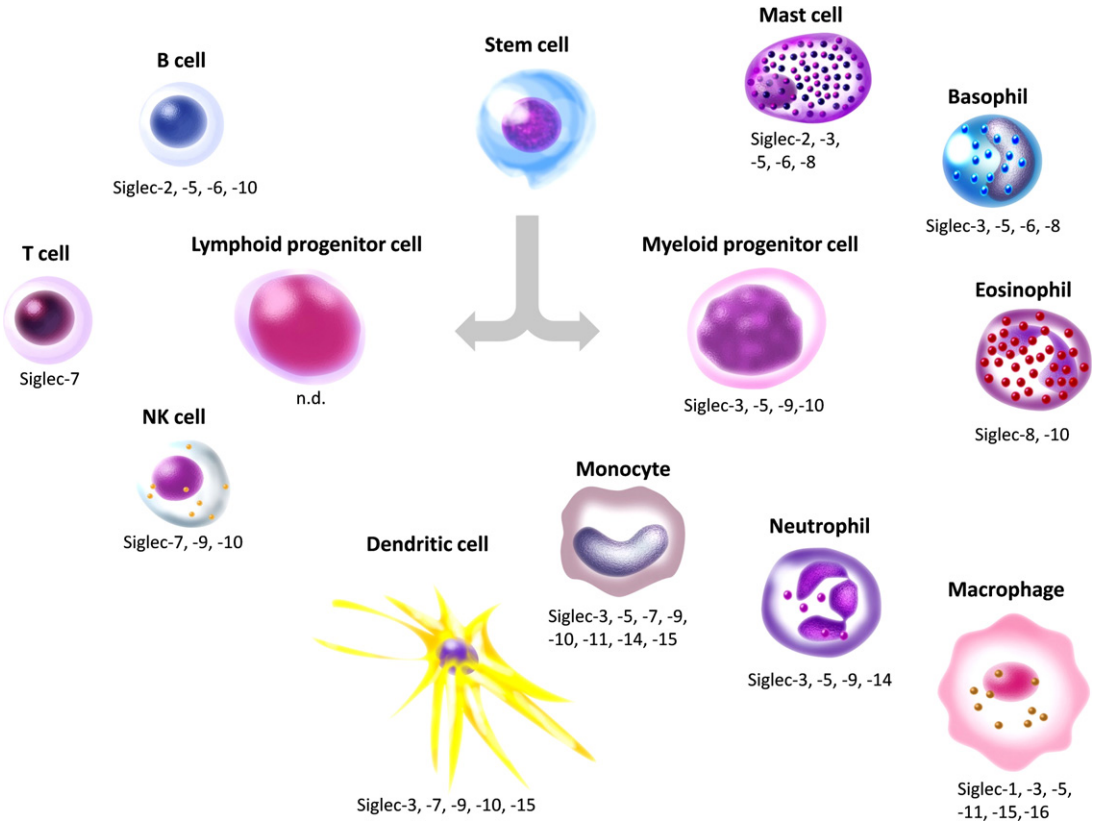

Fig. 2. Expression of Siglecs in haematopoietic and immune cells in humans. Shown is the current knowledge on the cell-type specific distribution of Siglecs in human haematopoietic and immune cells. Data are compiled from recent original work and reviews [4,72,114].
Art by Aldona von Gunten n.d. = not determined.

4. Siglec ligands

In contrast to the majority of human immune receptors that bind to protein ligands, Siglecs have the unique feature to specifically recognize sialylated carbohydrates, which together build a subclass of the glycome, called the "sialome". The sialome has been defined as the "total complement of sialic acid types and linkages and their mode of presentation on particular organelle, cell, tissue, organ or organism – as found at particular time and under specific conditions" [20]. Sialic acids are commonly found at the exposed termini of oligosaccharide chains attached to glycoproteins and glycolipids, most frequently as the non-reducing terminal molecules of N-glycans, O-glycans and glycosylphosphatidylinositol (GPI) anchored proteins or glycosphingolipids. Of importance, complexity of the sialome does reside not only in the large variety of possible modifications of sialic acid structure and linkages of the sialic acid to the underlying sugar, but also in the identity, arrangement and structural characteristics of these sugars, as well as spatial organization of the sialic acids at the cell surface.

The two major sialic acid core structures are 2-keto-3-deoxinonic acid (Kdn) and neuraminic acid (Neu), sharing nine carbons and differing at position C5. Two derivatives of Neu are the most common sialic acids found in nature: N-acetylneuraminic acid (Neu5Ac) and N-glycolylneuraminic acid (Neu5Gc), with 5,(7)9-N-O-diacetylneruaminic acid (Neu5,(7)9Ac_2) also being expressed but at lower levels by mammals. Generally, sialic acids are all based on a cyclic nine-carbon structure with a carboxylic acid group at position C1, which confers to these molecules an inherent negative charge. Multiple substitutions made at carbons C4, C7, C8 and C9 or at the amine at C5 result in a large variety of possible sialic acid structures. To date, more than 50 forms of naturally occurring sialic acids have been identified. Synthesis of sialic acid-containing carbohydrates is mediated by the action of different participating enzymes including sialyltransferases, that link sialic acids to subterminal oligosaccharides using α2–3 and α2–6 linkages or to other sialic acids using α2–8 and α2–9 linkages. In non-human vertebrates conversion of Neu5Ac to Neu5Gc occurs in the cytosol by the enzyme CMAH (CMP-N-acetylneuraminate monooxygenase). Of interest, due to mutations in the *CMAH* gene during human evolution, Neu5Gc is not present naturally in men, while it is a common sialic acid in other vertebrates [21]. This evolutionary loss of Neu5Gc synthesis capacity that occurred 2–3 million years ago in humans, might have contributed to the establishment of the observed differences in ligand binding and cellular expression pattern of some hominid Siglecs as compared to great apes and other species [3]. Exact relevance and potential implication in disease conditions of this human-specific changes in Siglec biology and sialic acid synthesis has not yet been fully elucidated and deserve further investigation.

Sialic acids and sialylation of glycoproteins and glycosphingolipids, respectively, have been associated in humans with normal development and immunity, intracellular signaling, cell–cell attachment, and host–pathogen interactions. Intriguingly, changes in sialic acid expression have been found in many human diseases including cancer and diabetes. However, still little is known on causes and consequences of these differences [3].

5. Siglec–sialoglycan interactions

Individual Siglec members recognize specifically a broad range of distinct and well-defined structures of sialic acid-containing carbohydrates [2]. Importantly, Siglec binding to sialic acids is not only largely influenced by the type of sialic acid (e.g. side chain, N-

acyl group and C-4 hydroxyl group), but also from its linkage to the subterminal sugars, the structure of more distal sugar residues as well as other modifications, such as sulphation. In this regard, by virtue of glycan array technology of the Consortium for Functional Glycomics (CFG), an initiative of the US National Institutes of Health (NIH), it has recently been shown that Siglec-9 binds specifically and with high affinity to the synthetic ligand 6-sulpho-sialyl-Le^x (data published on the CFG website http://www.functionalglycomics.org). Interestingly, sulphation of this same molecule at another site of the subterminal sugars (on the 6-hydroxyl of the galactose residue of SLe^x) leads to loss of binding to Siglec-9, while concomitant ability to be specifically recognized by Siglec-8. In addition to these binding characteristics, it has been demonstrated that treatment of target cells or glycoconjugates with broad specificity sialidases, known to release sialic acids from the underlying sugar chain, results in loss of glycan recognition by Siglecs, further arguing for a highly specific interaction of Siglecs with their sialic acid binding partner.

It is believed that Siglecs are able to recognize sialoglycans on the same cell surface glycocalyx (*in cis* interaction), where a local high concentration of sialic acids might be present [22]. This Siglec–ligands interaction appears to be the result of a dynamic equilibrium of binding to multiple sialoglycan ligands, in a low-affinity range, causing a "masking" of sialic acid binding sites. Under certain conditions, such as cell activation, the binding site may be released from binding *in cis* in a process called "unmasking", and results in availability for binding *in trans*, for instance, to sialoglycans expressed on surrounding cells, on soluble or attached glycoprotein and glycolipid structures, or on bacterial capsular polysaccharides. Alternatively, high-affinity engagement of Siglecs *in trans* may outcompete low-affinity interactions *in cis* [23]. One remarkable exception to this paradigm is Siglec-1, which is thought to be constitutively unmasked since it extends beyond the glycocalyx due to its large number of Ig repeats [22].

Until recently, the identity of specific ligands for Siglecs remained largely unknown and functional analysis of downstream effects of Siglecs' triggering mainly relied on their cross-linking with specific antibodies. Recently, glycan arrays have been developed that allow to screen for Siglec binding structures. Further advances in this field are expected by the use of a newly developed technique, the so-called "shotgun glycomics", an approach that allows direct study of complex glycomes isolated from animal cells and their functional and biologically relevant interactions with glycan-recognizing receptors, including Siglecs [24].

6. Evolution of CD33-related Siglec family members

As mentioned above, the CD33-related Siglec subfamily distinguishes itself from the conserved Siglec subgroup by its rapid evolution. Interestingly, however, comparison of evolution of Siglecs in mammals and in fish, shows that likely evolutionary conserved Siglecs (such as Siglec-1, -2, -4 and -15) have originally shared the same common ancestral region as CD33-related Siglecs, called "ancient Siglec cluster". With the exception of the fish, where Siglec-1 and -4 are still found closely associated to CD33-related Siglec genes, this ancient common Siglec locus got then dispersed on different chromosomes, in a species-specific manner [25].

Following comparative genomic analyses in different species including primates (human, chimpanzee, and rhesus macaque), rodents (rat and mouse), dog and marsupial (opossum), it has been recently proposed that the rapidly evolving CD33-related Siglecs derive from a primordial cluster that can be further subdivided into two defined subclusters, A and B. These subclusters were originally divided by a set of central non-Siglec genes [25]. After mammalian evolution 180 million years ago, the ancient rapidly evolving CD33-related Siglec subclusters have undergone multiple genetic processes, initiated by an inversed duplication step, which is believed to represent a key event in CD33-related Siglec gene evolution. This process was probably followed by further genetic evolution steps ranging from gene deletion, exon shuffling, pseudogenization, gene conversion between adjacent genes and pseudogenes to specific amino acid changes, predominantly in the amino-terminal sialic acid binding region of the receptor [26]. These evolutionary processes resulted in dramatic gene deletion in rodents, in particular in subcluster A, as opposed to an expansion of the original subclusters in primates. This observation implicates that the contracted CD33-related Siglec cluster present in mice and rats does not appear anymore to be the original cluster from which primate Siglecs were believed to evolve from, but rather the result of a large number of gene deletions. In contrast to rodents, primates express now a larger repertoire of Siglec genes, with the highest evolutionary pressure that seems to have affected the first Ig-like domain (Ig1) of the CD33-related Siglec receptors [26].

As mentioned above, novel, activating Siglecs have recently been identified, their origin still being under debate. Interestingly, these activating Siglecs, with the exception of Siglec-15 [27], share high homology in the extracellular domain and are paired with inhibitory Siglecs: Siglec-14 pairs with Siglec-5 [15], and Siglec-16 with Siglec-11 [28,29]. Based on this structural homology, it was postulated that evolutionary these activating Siglecs might arose as a way to counterbalance the inhibitory signals mediated by their paired inhibitory family members. It is possible that during evolution genes encoding for activating Siglecs have been transformed into pseudogenes to avoid the risk of an inappropriate and overwhelming immune activation [25].

Possible reasons for the rapid evolution observed in human CD33-related Siglec genes might be the attempt to adapt to evolutionary changes of their sialylated ligands expressed in nature (also known as "the sialome") [3] or alternatively, but not mutually exclusive, the need to protect the host from the attack of pathogens expressing diversified and changing sialic acids [25,26,29]. Indeed, pathogens have acquired various strategies to decorate their surface by sialic acid structures typical for vertebrates [30], and by this means might subvert innate immunity by molecular mimicry of host sialoglycans on their surface [31]. Thus, rapid evolution of CD33-related Siglecs might have been an effort from the host to prevent invasion by these microbes that incorporate host-like sialic acids and try in this way to behave like "self". The diversification of CD33-related Siglecs between mammals may therefore reflect the ongoing evolutionary arms race between host and pathogen [2,29,32], in a process also designated as Red Queen effect [2]. However, the practical consequence of the substantial difference in human versus rodent CD33-related Siglec evolution precludes in many instances comparative studies of Siglec expression and functions in human and rodents, as well as the use of mice as a suitable animal model for preclinical trials targeting these receptors.

7. Immune functions of human Siglecs

Despite major advances in the field, the exact function of the majority of human Siglecs has not yet been fully elucidated. To date, Siglecs have been implicated in the regulation of a broad variety of immune cellular activities with pleiotropic functions depending on the cell type. The fact that most Siglecs carry cytoplasmic signaling motifs suggests that upon ligation to high-affinity sialoglycans, multimerization and clustering of the receptors leads to intracellular signaling. Here, we provide a brief overview on documented Siglec functions (see also Fig. 3). For more detailed information on functions of individual Siglec members, the reader is referred to other excellent publications [2,4,12].

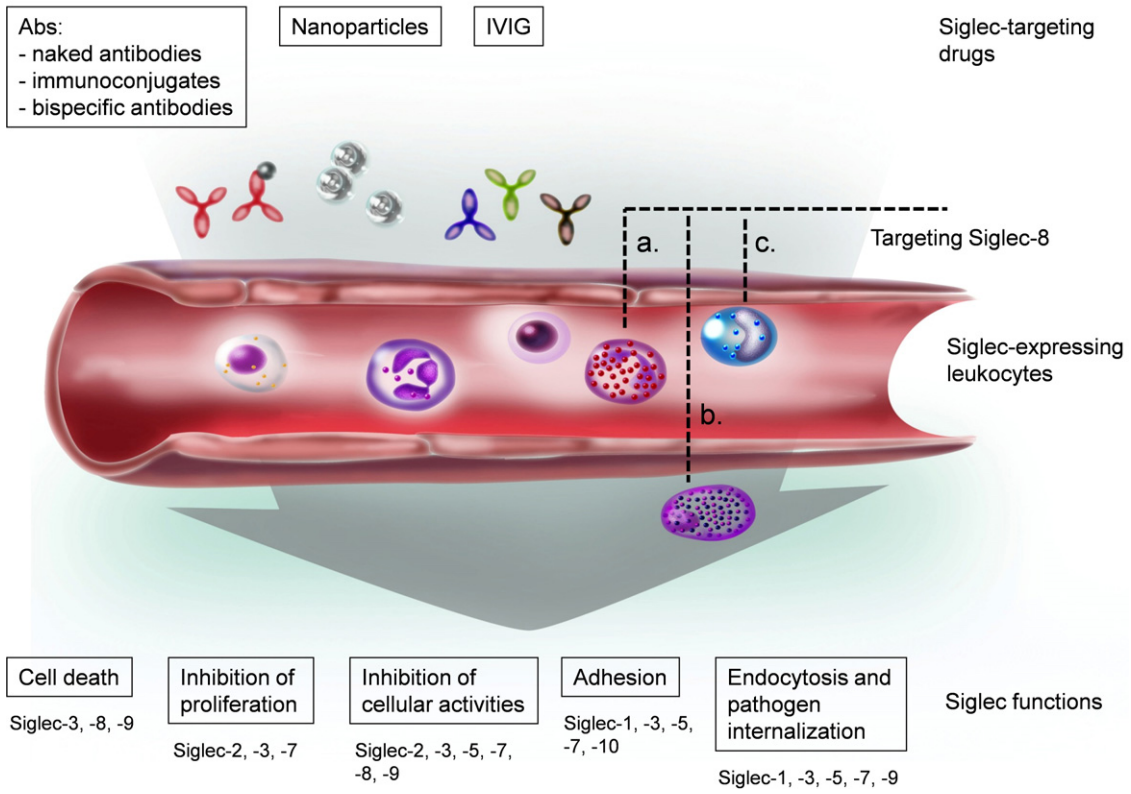

Fig. 3. Proposed therapeutic pathways by targeting Siglecs on immune cells. Engagement of Siglecs on immune cells initiates a range of functional activities, as discussed in the text for the indicated receptors. The cell type- and differentiation-dependent expression of Siglecs may be exploited for pharmacological targeting of a specific cell or a distinct set of cells for depletion therapies, or for modulation of cell functions by cross-linking or blocking agents. As an example, Siglec-8 is expressed on eosinophils (a), mast cells (b) and basophils (c), a group of cells that plays an important role in allergic diseases. Ongoing studies focus on the exploitation of Siglecs as targets of a broad range of therapeutic agents including antibodies, nanoparticles or intravenous immunoglobulin (IVIG).
Art by Aldona von Gunten.

7.1. Cellular adhesion, endocytosis and pathogen internalization

Various Siglec family members (e.g. Siglec-1, -3, -5, -7, -9 and -10) have been implicated in adhesion processes of human primary immune cells, malignant transformed cells or transfected cell lines [2,4]. Through binding *in trans* to high-affinity sialoglycans on target cells, Siglecs, either freely exposed on cell surfaces or upon release from binding *in cis* to low-affine ligands on the same cell surface (unmasking), favour cell–cell interactions. In such a manner, Siglecs could also interact with sialylated pathogens. Such pathogens, including bacteria (e.g. *Neisseria meningitides*, *Campylobacter jejuni*, *Trypanosoma cruzi* and others) and viruses (e.g. some respiratory viruses, HIV) are decorated on their surface with host-derived or self-synthesized sialoglycans [30]. Binding of these pathogen sialylated ligands to host Siglecs could either facilitate their endocytosis and clearance by host phagocytes, or as opposed, mediate their internalization to spread infection.

One outstanding example of a Siglec family member mediating cell–cell contact and endocytosis is Sialoadhesin (Siglec-1 or CD169). Sialoadhesin is a member of the evolutionary conserved Siglec subfamily and is specifically expressed on human macrophages: it is constitutively highly expressed on subsets of tissue-resident macrophages and can be rapidly upregulated by inflammatory macrophages upon activation [33]. With its unusual large number of immunoglobulin domains (17), Siglec-1 is the largest of the IgSF members. In contrast to most Siglecs, Siglec-1 lacks intracellular tyrosine based motifs suggesting that this receptor mediates cell–cell interactions, rather than intracellullar signaling. Siglec-1 has a preferential binding to α2–3-linkages, a terminal sequence frequently found in mammalian sialoglycans, but also on the surface of viruses and bacteria. In this context it has been suggested that pathogens, such as *Neisseria meningitides, Trypanosoma cruzi,* some respiratory viruses as well as HIV, engage Siglec-1 and exploit this receptor for internalization and spreading [34].

Pharmacologically, the endocytic function of Siglec molecules has been exploited for selective and targeted delivery of cell-directed therapies (see below).

7.2. Inhibition of cellular activities

One important role of Siglecs is their ability to regulate a large spectrum of cellular activities. Several Siglecs including the members Siglec-2, -3, -5, -7, -8 and -9 have been shown to be involved in inhibition of immune cell functions [4]. In lymphocytes, these capacities range from reduction of B cell activity and signaling (e.g. Siglec-2) [35], suppression of cytotoxicity and cytokine secretion in natural killer cells (e.g. Siglec-7) [36,37], as well as dampening of T cell signaling in Siglec-7 and Siglec-9 transfected Jurkat cells [38]. In the myeloid compartment, Siglec-8 triggering results in diminished release of inflammatory mediators by mast cells [39] (see below), CD33 (Siglec-3) engagement leads to reduced cytokine secretion by monocytes [40], and Siglec-5 or Siglec-7 transfection of rat basophilic leukemia cells (RBLs) inhibits FcεRI mediated calcium flux and serotonin liberation [41].

Functionally, CD22 is the probably most extensively investigated Siglec member, known to be implicated in the regulation of B cell signal transduction [42]. It is specifically expressed by B cells at the time of Ig gene rearrangement, and lost upon differentiation into plasma cells. It is well-established that CD22 is setting the threshold for B cell activation: upon ligation of the B cell receptor

(BCR) there is rapid phosphorylation of its ITIMs and consequent dampening of BCR signaling. Consistent with this observation, B cells from CD22-deficient mice exhibit hyperimmune responses [43]. Favoured sialic acid ligands for CD22 have been well documented: this receptor has high preference for α2–6-linked sialoglycans, which can be bound *in cis* directly on the B cell membrane. This ligation results in clustering of CD22 molecules in clathrin-coated pits and spatial segregation of CD22 from BCRs in resting cells [44]. Interactions with ligands *in trans* or cell activation might induce redistribution of CD22, colocalization with B cell receptors and negative influence on B cell activation and signaling. These observations have led to the exploitation of CD22 in cell-directed treatment strategies of B cell malignancies and autoimmune diseases, as discussed below.

7.3. Cell growth and survival

Siglecs (e.g. Siglec-2, -3, -7, -8 and -9) have been reported to regulate cell growth and survival, by both inhibition of proliferation and/or induction of apoptosis. For instance, CD22 triggering on B cells reduces proliferation [45], CD33 and Siglec-7 engagement on CD34+ myeloid progenitors as well as CD33 on monocyte-derived dendritic cells inhibits their growth [46]. Engagement of CD33 expressed on acute myeloid leukemia (AML) cells results in their apoptosis [47]. Ligation of Siglec-8 on eosinophils [7,48] or Siglec-9 on neutrophils [8] leads to their cell death, which is enhanced in the presence of pro-inflammatory cytokines, arguing for a delicate interplay between cytokine receptors and Siglecs on the surface of these cells [49]. Recruitment of alternative caspase-independent death pathways has been shown to be responsible for the increased cytokine-dependent death of neutrophils upon engagement of Siglec-9 [8].

Siglec-8, a member of the CD33-related Siglecs carries three Ig domains, is highly and selectively expressed by human eosinophils [50], by mast cells and at low levels by basophils [51]. *Siglec-8* gene can undergo alternative splicing, with resulting generation of two isoforms distinct in their intracellular domains [52]. Glycan-binding screening revealed that Siglec-8 display a unique binding specificity for the synthetic sulfated sialylated glycan 6′-sulfo-SLe^x [53]. For functional studies, Siglec-8 has been ligated either with monoclonal antibodies, in the presence of secondary cross-linkers, or auto-antibodies contained in commercial gamma globulin (IVIG) preparations [7,48,54]. This triggering leads to caspase-dependent apoptosis of eosinophils [48,54]. Interestingly, eosinophil death is further amplified by concomitant treatment of the cells with inflammatory cytokines (e.g. IL-5), normally known to favour eosinophil survival [7,54]. Similarly to successful attempts using anti-Siglec-F antibodies in mice (Siglec-F being the most close paralog of Siglec-8 in the murine system), this mechanism might be exploited to selectively target and deplete eosinophils *in vivo* with anti-Siglec-8 antibodies in disease conditions characterized by hypereosinophilia. In mast cells, Siglec-8 engagement has shown to significantly inhibit in an ITIM-dependent manner FcεRI mediated histamine and prostaglandine D2 release, calcium flux and anti-IgE-dependent bronchial smooth muscle contractions [39]. Intriguingly, genetic analysis of *Siglec-8* sequence variants revealed that some SNPs significantly associate with asthmatic conditions, suggesting that Siglec-8 might be a susceptibility locus for asthma. Altogether, these observations spur novel interest in the potential implications of Siglec polymorphisms in disease susceptibility [55].

7.4. Other functions of Siglecs

In a time where novel techniques for human cell isolation and cell function assessment are constantly improved, it is tempting to speculate that additional roles of human Siglecs in immune regulation will be identified and the yet relatively unexplored function(s) of certain family members (e.g. Siglec-6, -11, -14, -15 and -16) will be unravelled in coming years.

Siglec-10, another member of the CD33-related Siglec subfamily, has gained recent attention in view of its possible role in suppression of tissue-damage induced immune responses. Siglec-10 is expressed at low levels on several cells of the immune system, namely monocytes, macrophages, dendritic cells, a subpopulation of CD16+/CD56− cells, eosinophils and B cells [4,56]. A recent study on human Siglec-10 and mice Siglec-G demonstrated that ligation of these receptors to its newly identified ligand, sialylated CD24, act as negative regulators in host responses to danger-associated molecular patterns (DAMPs), which are abundantly released by damaged cells upon injury [57]. A variety of DAMPs (e.g. HMGB1, HSP70 and HSP90) were identified to associate with CD24 and to mediate negative signals through Siglec-G in the context of aseptic tissue injury. Remarkably, no dampening of immune responses against products of microbial origin (e.g. PAMPs) was documented, suggesting that this mechanism might help the host to discern infections from tissue injury [57]. Moreover, sialidases produced by microbes during infection are able to disrupt the CD24/Siglec-G inhibitory pathway, thus exacerbating pathogenicity in an animal model of polybacterial sepsis [58]. Finally, evidence for genetic alteration in CD24 [59] or sialic acid acetylesterases in autoimmune diseases [60] indicate that dysregulation of this pathway might be implicated and present a novel putative target for therapeutic approaches in these disorders.

8. Siglecs as targets of therapeutic antibodies

The discovery of hybridoma technology in 1975 [61] has initiated research and development of therapeutic monoclonal antibodies (mAbs) [62]. The success of rituximab, the first monoclonal antibody to be approved by the United States Food and Drug Administration (FDA) in 1997, has further stimulated rapid progress in the field during the last years [63]. Indeed, mAbs are increasingly being used for the treatment of different medical conditions including hematologic and nonhematologic malignancies [64–66], non-neoplastic hematologic diseases and autoimmune and inflammatory disorders [67–70]. Multiple agents have been developed from murine, chimeric to humanized antibodies [62], latter being less immunogenic in humans. Such mAbs are used ‘native’ (or ‘naked’) or conjugated with toxins, antitumor drugs, or radioisotopes [71]. Cell-directed depletion therapies with native antibodies involves different modes of action including antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC), or direct induction of apoptosis [72]. The selection of suitable target molecules is critical for the development of mAbs as therapeutic tools. Several features of Siglecs render these surface structures attractive targets for antibody-based immunotherapy: their cell type-specific expression on haematopoietic cells is exploited not only for depletion therapies in lymphomas and leukemia, but also for cell-directed therapies targeting immune cells as mediators of autoimmune and allergic diseases. Indeed, several antibodies targeting Siglecs are currently being tested in preclinical and clinical trials [72]. The endocytotic capacity of Siglecs is another property utilized for the cell-directed delivery of toxins or chemotherapeutics as immunoconjugates. Notably, dye-loaded nanoparticles decorated with anti-Siglec-7 antibodies have been shown to be readily internalized by Siglec-7 transfected mouse embryonic fibroblasts [73].

8.1. CD33-directed antibody therapy

CD33 (Siglec-3) was identified as a useful target antigen for acute myeloid leukemia (AML) therapy, as it is expressed on the cell surface

of the vast majority of patients with AML. Acute promyelocytic leukemia (APL) cells have a high expression of CD33, and a subset of patients with initially CD33 negative APL gains expression of CD33 at relapse, an observation that has clinical implications for immunotherapy [74]. CD33 is not found on tissues other than the haematopoietic system, where it is expressed at an earlier stage than other Siglecs [19,75]. HuM195, an unconjugated humanized anti-CD33 antibody has been shown to be rapidly internalized into target cells on binding the antigen [76]. Despite promising results in previous studies, in a subsequent phase III trial the treatment of myeloid leukemia with this naked antibody combined with chemotherapy showed no additional benefit [77]. In view of the endocytic capacity of CD33 the immunotoxin gemtuzumab, a calicheamicin-conjugated humanized murine anti-CD33 antibody, has been developed. In 2000, gemtuzumab ozogamicin (GO; Mylotarg; Wyeth, Madison, NJ) was approved by the FDA for the treatment of patients with CD33+ acute myeloid leukemia (AML) in first relapse who are 60 years of age or older and who are not considered candidates for other cytotoxic chemotherapies.

The recent Southwest Oncology Group (SWOG) 106 study, an open-label randomized phase III trial on the treatment of AML with Mylotarg in addition to chemotherapy, was stopped ahead of schedule based on the interim results showing no evidence of improved disease-free survival (DFS), and a fatal induction toxicity rate that was significantly higher in the study arm containing GO combined with induction chemotherapy than the arm using chemotherapy alone [78]. The results of this postapproval study were the basis for the recommendation by the FDA for withdrawal of GO from the market. However, Burnett et al. claim that the SWOG 106 trial may be confounded in that the dose of daunorubicin was lower in the GO treated arm, which might mask any benefit of GO in induction chemotherapy, and in that the mortality rate for the control arm was unexpectedly low [79]. Results from a similar study, the AML15 trial conducted by the United Kingdom Medical Research Council (MRC), were recently reported [79]. In this open-label trial including 1113 patients, the addition of GO to induction and/or consolidation chemotherapy in untreated younger patients has been evaluated. Although there was no overall difference in response or survival, distinct subsets of AML patients had improved survival with the addition of GO to induction chemotherapy: a significant benefit for patients with favorable cytogenetics, and a trend for benefit in intermediate-risk patients, but no benefit for patients with poor-risk disease [79]. In view of these results and the consideration that AML is a heterogeneous disease at the morphologic, molecular, and cytogenetic levels, the requirement of a more personalized treatment practice with the identification of subsets of patients best amenable to target-specific therapy becomes increasingly evident [80].

8.2. CD22-directed antibody therapy

CD22 (Siglec-2) is another Siglec member that is being pursued as a target of antibody therapy. Due to its B cell-restricted expression, it is considered an attractive target for immunotherapy of B-cell malignancies and autoimmune diseases [68,72]. Several anti-CD22 antibodies are currently being evaluated in clinical trials including the immunotoxins CM544 (conjugated to calicheamicin) and BL22 (conjugated to *Pseudomonas* exotoxin), as well as the naked antibody epratuzumab, a humanized version of the murine CD22-specific mAb LL2 [68,72,81]. Binding of epratuzumab to B cells results in internalization of cell surface CD22 within minutes rendering epratuzumab a promising agent for the design of immunoconjugates with toxins, cytotoxic drugs, or radionuclides [82]. In a B-lymphoma xenograft model system (90)Y-conjugated epratuzumab showed a therapeutic effect, which was further enhanced in combination with veltuzumab, an unconjugated humanized anti-CD20 IgG [83].

Several clinical studies showed favorable results for the therapeutic use of epratuzumab combined with rituximab (anti-CD20 mAb) for the treatment of non-Hodgkin lymphomas (NHLs) [84–86]. *In vitro*, epratuzumab and rituximab display different modes of action on Daudi Burkitt lymphoma cells in terms of cell growth inhibition, induction of apoptosis, and the ability to mediate CDC and ADCC [87]. Such different mechanisms of antitumor action may lead to additive or synergistic effects upon combination therapy with these antibodies and eventually help to overcome intrinsic single-antibody resistance. Since B cells play a central role of certain autoimmune diseases, targeting these cells by therapeutic antibodies to CD22 provides an interesting treatment option. Epratuzumab is currently being pursued in phase III clinical trials of systemic lupus erythematodus [88].

Novel classes of CD22-targeted immunotherapeutics are in development [72,89]. Following the encouraging results of combination therapy with the respective parental antibodies, multivalent and bispecific antibodies (bsAbs) have been generated through recombinant engineering from epratuzumab and veltuzumab (anti-CD20) [90–92]. Interestingly, such anti-CD20/22 bsAbs have been shown to be significantly more potent in killing lymphoma cells *in vitro* than their parental antibodies [90]. These bsAbs were effective at nanomolar concentrations and in the absence of a secondary cross-linking antibody. It is assumed that the multivalent binding capacity of these bsAbs leads to engagement of multiple signaling pathways and to an altered balance of pro- and antiapoptotic proteins, resulting in enhanced toxicity to B-cell lymphomas and leukemias [92]. As a consequence of simultaneously binding to CD20, the bsAb–antigen complex does not undergo endocytosis, despite engagement of CD22, probably leading to sustained CD22 signaling [90]. It is tempting to speculate that such pioneering work on CD22 will serve as a template for future therapeutic strategies targeting other Siglec members.

9. Siglecs as targets of intravenous immunoglobulin (IVIG)

Recently, we have reported the occurrence of functional antibodies specific for the receptors Siglec-8 [54] and Siglec-9 [93] in commercial intravenous immunoglobulin (IVIG) preparations. IVIG consists of pooled IgG from thousands of donors and is increasingly being used as a high-dose therapy for the treatment of autoimmune and chronic inflammatory diseases [94,95]. Currently, licensed applications for IVIG administration include Kawasaki disease, Guillain–Barré syndrome, idiopathic thrombocytopenia, and chronic inflammatory demyelinating polyneuropathy (CIDP), however IVIG is frequently used as an off-label application in a broad range of chronic inflammatory diseases [96]. The exact mode of its anti-inflammatory action still remains unclear, although multiple effects on humoral and cellular components of the immune system have been described [95,97]. In fact, depending on the pathogenesis of the disease specific mechanisms may act alone or in a concerted, mutually non-exclusive manner [95,98].

Since Siglec-8 and Siglec-9 are not expressed in rodents, initial functional studies on interaction of IVIG with these receptors have been performed *in vitro* using human primary cells. The use of human IVIG in conventional animal models entails the risk of loss-of-function or gain-of-function effects, and the study of IVIG *in vivo*, especially on Siglecs, must be addressed by carefully selected strategies including genetically altered animals. Generally, the analysis of IVIG in animal studies is hampered by the fact that IVIG is a human product and is expected to interact as a xenoprotein with the immune system of the animal host. It is unclear how concentration effects are translated into animal models, as in patients high-dose administration of IVIG is required to obtain

anti-inflammatory effects, at levels significantly above to those used for replacement therapy for the treatment of humoral immunodeficiencies.

IVIG induces cell death of granulocytes, an effect that might contribute to the clearance of these cells from the inflammatory site. In human granulocytes this effect is F(ab)-dependent and has been linked to the action of agonistic antibodies to Siglecs and Fas contained in IVIG. Cytokine-primed neutrophil and eosinophil granulocytes demonstrate a significantly higher death response to IVIG treatment compared to unstimulated cells *in vitro*, which is associated with the recruitment of caspase-independent death pathways. Similarly, eosinophils isolated from patients with hypereosinophilic syndrome, who presumably have been exposed to IL-5 *in vivo*, exhibit increased susceptibility for IVIG-mediated eosinophil death *ex vivo*. Upon blocking or depletion of anti-Siglec-8 antibodies the cytokine-dependent effect of IVIG is abolished and the death response of cytokine-primed eosinophils is comparable to unstimulated cells. In analogy, anti-Siglec-9 antibodies in IVIG seem to be responsible for the cytokine-dependent increment in death efficacy in neutrophils. The residual IVIG-mediated cytotoxicity in the absence of Siglec-8 or Siglec-9-specific autoantibodies may be attributed to the pro-apoptotic action of agonistic anti-Fas antibodies in IVIG, which have been shown to induce neutrophil death *in vitro* [99].

IgG in IVIG contains differential sialylation of the Fc core polysaccharides and Fc sialylation may contribute to the anti-inflammatory activity of IVIG as shown in certain animal models of inflammation [100]. These findings are discussed controversially [101]; for proof of concept future studies in a human system are required. Although the exact mechanisms of the reported phenomenon still remain to be elucidated, it has been suggested that instead of binding to Fc-gamma receptors, sialylated Fcs initiate an anti-inflammatory cascade through the lectin receptor SIGN-R1 or DC-SIGN [102,103]. Interestingly, the recognition of sialylated IVIG by the Siglec member CD22 has recently been reported [104]. The consequences of this interaction on B cell function remain to be shown. In contrast to CD22, sialylated IVIG is not recognized by Siglec-9 [105], and no reports of sialic acid-dependent binding of IVIG to other Siglec members are available to date.

IVIG contains antibodies so-called anti-idiotypes (Ids) that are capable of binding to variable regions of other antibody molecules to form Id-anti-Id complexes. Such Id-anti-Id complexes are suspected to contribute to dimer formation in IVIG. Indeed, we recently showed that dimeric IVIG contains anti-Siglec-9 autoantibodies and their anti-Ids [105]. It is possible that such Id-anti-Id interactions influence the activity of anti-Siglec-9 autoantibodies present in IVIG or in the patients' circulation [105].

10. Glycotargeting of Siglecs

Targeted drug or gene delivery (TDGD) has significant advantages over untargeted treatment modalities in terms of therapeutic efficiency and specificity. Carbohydrate recognition-based TDGD also called glycotargeting has been explored in the past decades [106], and recent advances in the fields of glycobiology and nanotechnology has led to the development of various novel carbohydrate-decorated drug and gene delivery systems [107]. Siglecs, as endogenous lectins, provide excellent targets for carbohydrate recognition-based TDGD given their selectivity for distinct carbohydrate ligands and their capacity to internalize bound carbohydrates. Besides the delivery of carbohydrate-decorated drugs, blocking or cross-linking approaches might be employed to either inhibit or trigger Siglec-specific functions in immune cells resulting in anti- or pro-inflammatory cellular responses, respectively. For instance, induction of eosinophil apoptosis [108] and inhibition of basophils and mast cells by cross-linking of Siglec-8 might become a potential pharmacological approach for treating allergic diseases or other eosinophil-associated disorders [109]. Indeed, it has recently been reported that cytokine-primed eosinophils undergo apoptosis *in vitro* upon multivalent binding to a synthetic Siglec-8 ligand, polymeric 6′-sulfated sialyl Lewis^x [110].

Multivalent presentation of high-affinity glycan ligands may be required for binding to Siglecs in order to generate sufficient avidity and to outcompete endogenous *cis* ligands on the target cell [23]. In fact, multivalent display of a high-affinity ligand of CD22 (^{BPC}NeuAc) on a polyacrylamide polymer resulted in binding to CD22 on native B cells *in vitro*. A synthetic heterobifunctional molecule comprising the ligand ^{BPC}NeuAc coupled to the antigen nitrophenol (NP) could use anti-NP antibody as a scaffold to efficiently assemble IgM-CD22 complexes on the surface of native B cells [111]. Among anti-NP antibodies IgM as a decavalent protein scaffold was more efficient than tetravalent IgA or bivalent IgG.

In an elegant recent study, doxorubicin-loaded liposomal nanoparticles decorated with the high-affinity CD22 ligand ^{BPC}NeuAc have been evaluated for targeting of B lymphoma cells [112]. These targeted liposomes were actively bound and endocytosed by B cells and able to kill primary malignant B cells from patients with hairy cell leukemia, marginal zone lymphoma, and chronic lymphocytic leukemia. Moreover, these CD22-targeted liposomes significantly extended survival in a xenograft model of disseminated Daudi human B-cell lymphoma.

Taken together, these discussed studies provide the proof-of-concept that glycotargeting of Siglecs can be exploited for therapeutic applications. In addition, insights from pioneering work on CD22 and Siglec-8, together with advances in nanotechnology, may have important implications for the design of novel therapeutics for glycotargeting also of other Siglec members.

11. Conclusions

In the recent years, a significant body of evidence has accumulated suggesting that Siglecs play an important role in the regulation of the immune system. Furthermore, their cell-specific expression and endocytotic capacity renders them suitable molecules for targeted drug delivery. Besides native, conjugated or engineered antibodies, novel therapeutic strategies are currently being investigated that take advantage of the selective carbohydrate binding properties of this only recently defined family of lectins. Advances in nanotechnology might also contribute to a rational design of biodegradable drugs that provide a robust scaffold under physiological conditions and allow for multivalent, high-avidity interactions between Siglecs and their specific carbohydrate ligands. An increasing body of experimental evidence suggests that antibody-based or glycotargeting strategies might be therapeutically employed to either induce or inhibit Siglec signaling in immune cells, e.g. by cross-linking or blocking of the ligand binding site. Such insights might lead to the development of both agonistic and antagonistic Siglec-targeting drugs that can be used for suppression or activation of defined subsets of immune cells depending on the receptor expression. The collective effort of pharmacologists, glycobiologists, chemists and clinicians is required to successfully translate emerging evidence on Siglecs into the development of novel drugs for the therapy of autoimmune and chronic inflammatory disease, as well as cancer.

Conflict of interest

The authors declare no conflict of interest.

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