Published in final edited form as:

Pharmacol Ther. 2012 September ; 135(3): 327–336. doi:10.1016/j.pharmthera.2012.06.005.

Siglec-8 as a drugable target to treat eosinophil and mast cell-associated conditions

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Abstract

Siglecs (sialic acid immunoglobulin-like lectins) are members of the immunoglobulin gene family that contain sialoside binding N-terminal domains. They are cell surface proteins found predominantly on cells of the immune system. Among them, Siglec-8 is uniquely expressed by human eosinophils and mast cells, as well as basophils. Engaging this structure with antibodies or glycan ligands results in apoptosis in human eosinophils and inhibition of release of preformed and newly generated mediators from human mast cells without affecting their survival. Pro-apoptotic effects are also seen when its closest functional paralog, Siglec-F, on mouse eosinophils is similarly engaged in vitro, and beneficial effects are observed after administration of Siglec-F antibody using models of eosinophilic pulmonary and gastrointestinal inflammation in vivo. Siglec-8 targeting may thus provide a means to specifically inhibit or deplete these cell types. Cell-directed therapies are increasingly sought after by the pharmaceutical industry for their potential to reduce side effects and increase safety. The challenge is to identify suitable targets on the cell type of interest, and selectively deliver a therapeutic agent. By targeting Siglec-8, monoclonal antibodies and glycan ligand-conjugated nanoparticles may be ideally suited for treatment of eosinophil and mast cell-related diseases, such as asthma, chronic rhinosinusitis, chronic urticaria, hypereosinophilic syndromes, mast cell and eosinophil malignancies and eosinophilic gastrointestinal disorders.

Keywords

Siglec-8; Siglec-F; Glycan ligands; Allergic diseases; Apoptosis; Liposomes

1. Introduction

Siglecs (sialic acid immunoglobulin-like lectins) are members of the immunoglobulin (Ig) gene family that contain sialoside binding N-terminal domains (Crocker et al., 1998). They are cell surface proteins found predominantly on cells of the immune system. Siglec-8 was

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Conflict of Interest statement

Dr. Bochner is a co-inventor on existing and pending Siglec-8-related patents. Dr. Bochner may be entitled to a share of royalties received by the University on the potential sales of such products. Dr. Bochner is also a co-founder of, and owns stock in, Allakos, Inc., which makes him subject to certain restrictions under University policy. The terms of this arrangement are being managed by the Johns Hopkins University in accordance with its conflict of interest policies. Drs. Paulson and Kawasaki are inventors on patent applications managed by The Scripps Research Institute relating to the use of siglec ligands for targeting siglec-bearing cells, and they may be entitled to a share of license revenues in the event that they are realized.

originally identified as being uniquely expressed on human eosinophils, mast cells and weakly on basophils (Floyd et al., 2000; Kikly et al., 2000). These cells express other siglecs as well (von Gunten & Bochner, 2008; Bochner, 2009; Castro et al., 2011), but it is their selective expression of Siglec-8 that provides a unique opportunity to exploit this molecule for targeting of these cells in diseases in which the biology of these cells is excessive and unwanted, such as in asthma, chronic rhinosinusitis, chronic urticaria, hypereosinophilic syndromes, mast cell and eosinophil malignancies and eosinophilic gastrointestinal disorders.

After presenting an overview of siglecs, their cellular expression patterns and sialylated glycan ligands, the biology of Siglec-8 and its closest functional paralog in the mouse, Siglec-F, will be reviewed, with a focus on mechanisms and cellular consequences that occur following Siglec-8 or Siglec-F engagement on an eosinophil or mast cell (unfortunately nothing is known for the basophil), along with an account of our current understanding of their endogenous tissue ligands. Finally, a description and comparison of glycan and antibody-based approaches occurring via endocytic mechanisms will be discussed as strategies to be used to therapeutically target eosinophils and mast cells via their preferential expression of Siglec-8. The broad concept of therapeutic targeting of siglecs has been the subject of a number of excellent recent publications including those cited here (Varki & Angata, 2006; Crocker et al., 2007; McMillan & Crocker, 2008; von Gunten & Bochner, 2008; O'Reilly & Paulson, 2009; Chen et al., 2010; Magesh et al., 2011; Spergel et al., 2011).

2. Siglec structures and patterns of expression

All siglecs are single-pass transmembrane proteins belonging to the Ig superfamily with I-type lectin domains in their N-terminal regions (Powell & Varki, 1995; Varki & Angata, 2006). Figs. 1 and 2 display the structures of human and mouse siglecs, respectively (reproduced from (S. von Gunten & Bochner, 2008)). Attached to the lectin domain are 1 to 15 Ig domains, a transmembrane domain and a cytosolic tail (Varki & Angata, 2006). Siglecs can be grouped into two types based on evolutionary and sequence similarities: the Siglec-3 (cluster of differentiation [CD]33)-related siglecs (Siglec-5 [CD170], Siglec-6 [CD327], Siglec-7 [CD328], Siglec-8, Siglec-9, Siglec-10, Siglec-11, Siglec-14 and Siglec-16) and all others (Crocker et al., 2007; Varki, 2010). CD33-related siglecs in humans are exceptionally related in structure but are evolving away from other mammalian siglecs (Cao et al., 2009; Varki, 2010). Mouse cells can express CD33 but the murine genome possesses only about half as many CD33-related siglecs (Siglec-E, -F, -G and -H) and they represent paralogs, not true orthologs, of human siglecs. Siglec-H lacks any tyrosine signaling motifs in its cytoplasmic tail; whether it can bind sialic acid remains controversial (Avril et al., 2006; Blasius et al., 2006; Takagi et al., 2011).

In contrast, sialoadhesin (Siglec-1 or CD169), CD22 (Siglec-2), myelin-associated glycoprotein (Siglec-4, found on nerve-related cells rather than leukocytes) and Siglec-15, are the only true orthologs of their human counterparts (Angata et al., 2004; Crocker, 2005; Varki & Angata, 2006; Crocker & Redelinghuys, 2008). The genes encoding for all human siglecs are located on human chromosome 19q13.3–q13.4, except for sialoadhesin (chromosome 20) and Siglec-15 (chromosome 18). Siglec-12 in humans cannot bind sialic acid and thus is not a true siglec, so it has been given the designation Siglec-XII. Siglec-13 does not exist on human leukocytes but can be identified on baboon and chimpanzee cells (Angata et al., 2004; Cao et al., 2009).

Most siglecs have immunoreceptor tyrosine-based motifs (ITIMs) located in their intracellular domain, suggesting that these receptors are involved in negative cell signaling

(Crocker, 2002, 2005; Varki & Angata, 2006; Crocker et al., 2007; Varki, 2007; von Gunten & Bochner, 2008, 2009). The best studied is the conserved membrane proximal ITIM motif containing the amino acid sequence (I/L/V) xYxx(L/V) (Vely & Vivier, 1997). ITIMs in various siglecs can recruit Src homology 2 domain-containing phosphatase (SHP)-1 and others when phosphorylated (Ikehara et al., 2004), and inhibit cell function when cross-linked (Avril et al., 2006). It is therefore assumed that engagement of these ITIM-containing siglecs results in the delivery of a negative regulatory signal and in some cases this signal is apoptosis. Exceptions to the cytoplasmic ITIM paradigm include Siglec-1 and Siglec-4, where no such ITIMs exists, and Siglecs 14 and 15, where no such ITIMs exists but instead these siglecs co-associate with DAP-12 and therefore have activating activity. CD33-related siglecs also possess a second membrane-distal tyrosine motif. Although little is known about its function, these appear to be immunoreceptor tyrosine-based switch motifs that may recruit so-called adaptor molecules like signaling lymphocytic activation molecule-associated protein or other molecules (Munitz & Levi-Schaffer, 2007; Shik & Munitz, 2010). More work is needed to explore this further.

Many human cells express siglecs and each siglec has a unique pattern of expression as shown in Table 1. Other than Siglec-4, found on cells of the nervous system, Siglec-6, found on placental trophoblasts (Brinkman-Van der Linden et al., 2007), and Siglec-XII, found on epithelial and other cells (Angata et al., 2001b), all are expressed by various leukocyte subsets and mast cells. Some siglecs are more distinctively expressed than others. For instance, macrophages uniquely express sialoadhesin (Siglec-1, true in the mouse too) (Hartnell et al., 2001), B cells uniquely express CD22 (Siglec-2, also true in mice), and eosinophils and mast cells uniquely express Siglec-8, although for mast cells, Siglec-6 is particularly prominent in its expression (Florian et al., 2006; Yokoi et al., 2006). Among the CD33-related siglecs, Siglec-E is found on mouse neutrophils, monocytes, and dendritic cells and closely resembles Siglec-9, whereas Siglec-F and Siglec-G are considered the closest functional paralogs of Siglec-8 and Siglec-10, respectively (Aizawa et al., 2003; Zhang et al., 2004; Tateno et al., 2005; Hoffmann et al., 2007). Siglec-H is found on plasmacytoid dendritic cells but to date has no known human counterpart (Blasius et al., 2006; Zhang et al., 2006). CD33-related siglecs are roughly 50% identical, yet cellular expression patterns are not necessarily the same from species to species (e.g., see below regarding patterns of Siglec-8 versus Siglec-F expression). Because human CD4⁺ lymphocytes have evolved to lack them (Nguyen et al., 2006), siglecs are often thought of as innate immune receptors (Crocker, 2005), although CD8⁺ T cells can express Siglec-7 and Siglec-9 (Ikehara et al., 2004), and under allergic inflammatory conditions, Siglec-F can reportedly be detected at low levels on CD4⁺ T cells (Zhang et al., 2007).

3. Expression patterns of Siglec-8 and Siglec-F during hematopoiesis, across various species and in disease states

Siglec-8, originally discovered by two laboratories using the same eosinophil cDNA library generated from a patient with marked peripheral blood eosinophilia, was initially described as having a short cytoplasmic domain lacking the tyrosine motifs found in other CD33-related siglecs (Floyd et al., 2000; Kikly et al., 2000). In its original description it was also referred to as SAF-2 or sialoadhesin factor 2. It soon became clear that mast cells and basophils also expressed Siglec-8, and this was followed by the observation that Siglec-8 could undergo alternative splicing to yield both a “short form” as originally described, and a “long form” containing two tyrosine cytoplasmic motifs consistent with other CD33-related siglecs (Foussias et al., 2000; Aizawa et al., 2002). Since then, the term Siglec-8 is used to describe the “long form”. Additional studies examining leukocyte transcriptomes in an unbiased fashion confirmed the highly selective expression of Siglec-8 by eosinophils (Liu et al., 2006).

Relatively little is known about Siglec-8 expression during inflammation and disease states, and what is known pertains only to eosinophils as nothing is known about mast cells on this topic. Eosinophils obtained by bronchoalveolar lavage from asthmatic subjects have similar levels of surface Siglec-8 when compared to their blood counterparts (unpublished observations). In one patient with hypereosinophilic syndrome, both the levels and function of Siglec-8 on their eosinophils appeared normal despite the presence of a point mutation near the putative glycan binding site (Gao et al., 2010). A limited analysis of various human blood samples revealed that Siglec-8 is expressed at normal levels on eosinophils and basophils from subjects with chronic eosinophilic and chronic myelogenous leukemia, and on normal bone marrow mast cells and mast cells from subjects with indolent systemic mastocytosis (Hudson et al., 2011). As predicted by genomic analyses (Angata et al., 2004; Cao et al., 2009), Siglec-8 is maintained in chimpanzees but is not detected on baboon, rhesus and cynomolgus monkey eosinophils or dog mastocytoma cells when analyzed by indirect immunofluorescence and flow cytometry (Hudson et al., 2011). So far, there is no known eosinophil-like cell line that expresses Siglec-8 (Kikly et al., 2000; Hudson et al., 2011), but several mast cell lines, such as LAD2, LUVA, and HMC1.2 (but not HMC1.1) do express Siglec-8 (Yokoi et al., 2006; Hudson et al., 2011; Salicru et al., 2012) (Table 2).

Neither Siglec-8 nor Siglec-F is expressed on stem cells, and based on studies where cells are grown in vitro from precursors, Siglec-8 appears late in mast cell and eosinophil maturation, as is the case for Siglec-F on mouse eosinophils (Kikly et al., 2000; Yokoi et al., 2006; Dyer et al., 2008; Hudson et al., 2011). The cellular expression pattern of Siglec-F is slightly different from that of Siglec-8. Siglec-F was initially identified in immature cells of the myelomonocytic lineage and in a subset of CD11b-positive cells in some tissues (Angata et al., 2001a). Using interleukin (IL)-5 transgenic mice and Northern blotting analyses, mRNA levels of Siglec-F were found to be present in mouse eosinophils (Aizawa et al., 2003). Additional studies using antibodies, Siglec-F null mice and other molecular analyses have confirmed selective Siglec-F expression by eosinophils (Zhang et al., 2004; Tateno et al., 2005; Zhang et al., 2007). Because the sequence of Siglec-F is more similar to that of human Siglec-5, which is not expressed by human eosinophils, this was rather unexpected (Aizawa et al., 2003). Subsequent studies showed that Siglec-F is expressed on a wider range of cells than Siglec-8, including mouse alveolar macrophages, some T cells, and weakly on neutrophils (see Table 2) (Stevens et al., 2007; Tateno et al., 2007; Zhang et al., 2007).

4. Glycan ligands for Siglec-F and Siglec-8

As implied by their name, siglecs recognize sialylated glycans as ligands. Numerous groups have investigated the sialic acid-dependent binding of siglecs, and have demonstrated that each siglec exhibits preference for one or several of the diverse glycan structures found on glycans of glycoproteins and glycolipids (Campanero-Rhodes et al., 2006; Crocker et al., 2007; Varki, 2009; Paulson et al., 2012). Although there is an extensive literature on siglec ligands, a family-wide comparison is difficult because of the diverse assays used to analyze ligand specificity. Nonetheless, it is abundantly clear that siglecs exhibit divergent specificities for their glycan ligands, which play a role in their biology through their interactions with ligands expressed on the same cell in *cis* and on other cells and/or soluble glycoconjugates in *trans*.

Although some siglecs exhibit quite broad specificity for sialosides commonly found on glycans of glycoproteins and glycolipids, others exhibit a strong preference for one or several sialoside structures. Siglec-8 and the murine paralog Siglec-F are among the most specific (Bochner et al., 2005; Tateno et al., 2005). As illustrated in Table 3, Siglec-8 and Siglec-F bind with highest affinity to two sialosides that have in common a terminal epitope

with both sialic acid and sulfate attached to the same galactose residue (NeuAc α 2-3(6S)Gal β 1-4($\pm$ Fuc α 1-3)GlcNAc-). Of the two, Siglec-8 is the most specific, while Siglec-F exhibits weaker affinity for non-sulfated glycans. No other human or mouse siglec exhibits a strong preference for this ligand, as illustrated by the exemplary siglecs in Table 3. However, Rinaldi and coworkers, using arrays of glycolipids, immobilized either individually or in complexes on either a polystyrene surface or polyvinylidene difluoride membrane, reported that Siglec-F-Fc, a fusion protein containing two extracellular Siglec-F domains linked to the human Fc (fragment crystallizable) region of IgG) unexpectedly bound to the ganglioside GM2 and a mixture of GM2:GT1b but only on the polyvinylidene difluoride membrane, suggesting that the surface on which a siglec ligand is displayed, and what it is displayed with (i.e., individually or mixed with other glycolipids) can influence binding patterns (Rinaldi et al., 2009). In contrast to Siglec-8, Siglec-9 preferentially recognizes a related sialoside with sulfate on the 6-position of the GlcNAc (Table 3). Similarly, human CD22 preferentially recognizes yet another related sialoside that differs in the sialic acid linkage and position of the sulfate (NeuAc α 2-6Gal β 1-4(6S)GlcNAc).

Knowledge of the ligand specificity of a siglec does not directly inform us about the ligands encountered in situ, but the information provides a concrete basis for experimentation to identify the biologically relevant ligands. The ligands for human and murine CD22 provide good examples of this point. Using an antibody specific for the preferred ligand of human CD22, Kannagi and coworkers showed that this ligand is highly expressed on naïve human B cells, and is the major biologically relevant ligand for CD22 (Kimura et al., 2007), yet germinal center B cells no longer express this ligand, suggesting that there is active regulation of the biosynthesis of the CD22 ligand in vivo. The challenge for the future will be to systematically compare the specificities of all members of the siglec family, including Siglec-8, using the same assay systems, and to determine the extent to which the specificity of each siglec has on the biology it mediates.

5. Endogenous tissue ligands for Siglec-F and Siglec-8

Although the exact composition of natural tissue ligands of Siglec-F and Siglec-8 are still under study, immunohistochemical approaches have begun to reveal the potential tissue distribution of these ligands. For example, Siglec-F-Fc has been used to screen for tissue ligands, and was found to selectively bind mouse lung epithelium and goblet cells with the staining being eliminated by mutating the arginine in the Siglec-F-Fc putative glycan binding domain, or by pretreating the lung tissue with Maackia amurensis lectin (recognizing α 2,3-linked sialic acid), sialidase, periodate or proteinase K (Zhang et al., 2007; Patnode et al., 2009; Guo et al., 2011). The staining intensity for this lung epithelial Siglec-F-Fc binding material was increased following allergic lung inflammation in mice and sheep (Zhang et al., 2007; Patnode et al., 2009; Cho et al., 2010). Interestingly, some peribronchial cells, glands and mononuclear leucocytes seem to express Siglec-F-Fc binding molecules under conditions of allergic airway inflammation, but it is not known if these differ from those detected on the epithelium (Zhang et al., 2007; Patnode et al., 2009). Increases in Siglec-F-Fc binding to airway epithelium were also observed after IL-4 or IL-13 stimulation, but not other cytokines such as TNF- α , in vivo and in vitro (Cho et al., 2010; Kiwamoto et al., 2011a, 2011b). Lungs from *St3gal3* null mice, but not *St3gal2* null mice, were nearly devoid of Siglec-F-Fc staining (Guo et al., 2011), implicating the *St3gal3* sialyltransferase in the generation of Siglec-F ligands. Similar sialidase-sensitive Siglec-F binding molecules were observed in preliminary studies using material derived from mouse primary tracheal epithelial cells (mTECs) and culture supernatants (Cho et al., 2010; Kiwamoto et al., 2011a, 2011b). These results suggest that this Siglec-F-Fc binding molecule is an α 2,3-linked sialic acid-containing glycoprotein that increases under allergic inflammatory conditions. Further support for this conclusion comes from early studies

reporting that pre-incubation of mouse lung tissue sections or mTEC with a novel anti-6'-sulfo-sLe^x IgY antiserum blocked Siglec-F-Fc binding (Kiwamoto et al., 2011b, 2012). Although further experimentation is required, this lung epithelial Siglec-F-Fc binding material likely represents a Siglec-F natural tissue ligand that contains the 6'-sulfo-sLe^x structure.

In contrast, Siglec-8-Fc did not bind human lung epithelium in tissue sections but instead bound human airway glands and was poorly blocked by anti-6'-sulfo-sLe^x IgY (Kiwamoto et al., 2012). Western blotting using Siglec-8-Fc recognized a ≈500 kDa sialidase sensitive band from supernatants of human bronchial explants (Kiwamoto et al., 2012). Although both Siglec-F-Fc and Siglec-8-Fc detected sialidase-sensitive high molecular weight (≈460–500 kDa) material from both species of lung, a lower molecular weight band (≈225 kDa) was detected only in the mTEC lysate using Siglec-F-Fc (Kiwamoto et al., 2012). Based on these early results from ongoing studies, lung ligands for Siglec-F and Siglec-8 may be different in their location of expression and perhaps in their glycan composition as well. Work is underway to purify and fully biochemically characterize glycoprotein ligands for Siglec-F and Siglec-8 (<http://lidpeg.jhmi.edu/>). Until these lung-derived candidate ligands are more fully characterized biochemically, it remains unclear whether mouse and human ligands for Siglec-F and Siglec-8, respectively, are the same or different. Future studies using sialyltransferase and sulfotransferase deficient mice should also help to determine the functional contribution of these enzymes to the generation of natural Siglec-F tissue ligands under normal and inflammatory conditions.

6. Targeting Siglec-F and Siglec-8 via antibodies and glycan ligands

6.1. Anti-eosinophil properties

As introduced above, Siglec-8 expressed on eosinophils has potential as targets for cell-directed therapeutics in a variety of diseases because ligation of Siglec-8 on eosinophils with monoclonal antibodies (mAbs) cause caspase and/or reactive oxygen species (ROS) dependent apoptosis of the cell. This property is amplified under conditions of eosinophil activation such as cytokine priming with IL-5, granulocyte macrophage colony-stimulating factor or IL-33 where ROS dependent mechanisms along with mitochondrial injury are predominantly if not exclusively involved (Nutku et al., 2003, 2005; von Gunten et al., 2007; Nutku-Bilir et al., 2008; Na et al., 2011).

The fact that antibody engagement of Siglec-8 on eosinophils causes their apoptosis suggested that engagement of Siglec-8 with glycan ligands could have similar effects. Indeed, a soluble polymer displaying the glycan 6'-sulfo-sLe^x selectively bound to human eosinophils and HEK 293 cells expressing Siglec-8 (Hudson et al., 2009). Polymer binding was inhibited by a polyclonal anti-Siglec-8 antibody. In whole human blood, eosinophils were the only leukocyte subtype to detectably bind polymeric 6'-sulfo-sLe^x. IL-5-primed eosinophils underwent apoptosis when incubated with either anti-Siglec-8 mAb or polymeric 6'-sulfo-sLe^x, although the glycan polymer was less effective. These data were the first to demonstrate that a soluble, multivalent polymer decorated with a unique glycan, 6'-sulfo-sLe^x, selectively binds to human eosinophils and induces their apoptosis. These results provide proof-of-concept that glycan-based approaches can be used to selectively target eosinophils and could be exploited as it relates to glycan nanoparticle targeting.

Several reports have documented that in vivo administration of monoclonal and polyclonal antibodies recognizing Siglec-F reduces blood and tissue eosinophils in vivo, including models of hypereosinophilia and eosinophilic leukemia, and reduces signs and sequelae of eosinophil-mediated lung and intestinal inflammation in murine models (Zimmermann et al., 2008; Song et al., 2009a, 2009b). Furthermore, mice deficient in Siglec-F show exaggerated

eosinophilic inflammation and tissue remodeling in asthma models, but there was no significant effect on airways hyperreactivity (Kearley et al., 2007; Zhang et al., 2007; Cho et al., 2010). For example, in a mouse model of gastrointestinal eosinophilia and diarrhea, Siglec-F mAb administration normalized tissue eosinophil levels and weight gain (Song et al., 2009b). In a subsequent paper, this same group demonstrated that mice sensitized to ovalbumin and challenged via the airway repetitively for 1 month while being given anti-Siglec-F not only showed decreased airways eosinophilia, but almost complete normalization of sub-epithelial fibrosis, a reduction in mucus cells and smooth muscle layer thickness, and a significant reduction in airway cells positive for the profibrogenic cytokine transforming growth factor- β (Song et al., 2009a). These data provide encouragement in support of targeting an eosinophil-selective siglec because of the profound effect on eosinophil numbers, tissue injury and disease activity.

Although the exact signaling mechanisms involved in Siglec-8 and Siglec-F induced eosinophil death are not yet delineated, ongoing work is employing mouse strains deficient in key intracellular molecules to test the roles of phosphatases (e.g., using SHP-1 null mice) and ROS (e.g., using p47 NADPH oxidase deficient mice) in Siglec-F signal transduction using eosinophils grown from bone marrow precursors from such mice. Data generated so far surprisingly suggest neither SHP-1 nor ROS is required for Siglec-F-induced apoptosis, and indeed unlike what is seen with human eosinophils activated via Siglec-8, mouse eosinophils activated via Siglec-F do not produce any detectable ROS (unpublished observations). Recent acquisition of inhibitors of molecules that may be downstream of Siglec-8/-F signaling should allow the comparison of effects in mouse versus human eosinophils, and so far, ERK phosphorylation has been implicated in the IL-5-enhanced apoptosis seen with Siglec-8 and human eosinophils (Kano et al., 2012).

6.2. Anti-mast cell properties

In a separate series of experiments, the hypothesis that activation via Siglec-8 will affect the survival and/or secretory functions of mast cells was tested. Studies using human mast cells generated from CD34⁺ precursors showed that Siglec-8 engagement failed to induce apoptosis (Yokoi et al., 2008). However, pre-incubation with Siglec-8 mAbs significantly inhibited Fc ϵ RI-dependent histamine and PGD₂ release from purified mast cells, shifting the dose response curves to stimulation with anti-Fc ϵ RI antibody by 1–2 logs. Also significantly inhibited was mast cell-dependent contraction of human bronchial smooth muscle rings in vitro. Further studies with mast cell and Siglec-8 transfectants revealed that preincubation with Siglec-8 mAbs inhibited Fc ϵ RI-dependent Ca⁺⁺ flux and release of β -hexosaminidase that required the presence of the membrane-proximal ITIM tyrosine residue. Thus, although mast cells seem to behave differently than eosinophils following Siglec-8 engagement with respect to apoptosis, both cells display inhibitory responses that fit with a favorable clinical effect profile when considered as treatment for mast cell and eosinophil-related disorders. Unfortunately, Siglec-F is not expressed on mouse mast cells, so its function on these cells cannot easily be determined.

7. Prospects for therapeutic applications of targeting siglecs, including Siglec-8

The fact that siglecs exhibit highly restricted expression in selected hematopoietic cells and oligodendrocytes has drawn attention to their potential as targets for development of cell directed therapies (von Gunten & Bochner, 2008; O'Reilly & Paulson, 2009; Farid et al., 2012). Indeed, CD22 (Siglec-2) and CD33 (Siglec-3) were originally discovered as markers of B cell lymphomas and acute myeloid leukemias, respectively, and soon became targets for the development of immunotherapies to treat these diseases (Ball, 1988; Kreitman &

Pastan, 2006). Because many siglecs are endocytic receptors (Shan & Press, 1995; Crocker et al., 2007), immunotoxins have been adopted as an attractive clinical strategy since binding of the antibody to CD22 and CD33 results in endocytosis of the complex, delivering the toxin into the cell. Immunotoxins targeting both CD22 and CD33 continue to be actively pursued in clinical trials for lymphomas and leukemias expressing these siglecs (Kreitman & Pastan, 2006; Tu et al., 2011; Jurecic, 2012; Kreitman et al., 2012; Ogura et al., 2012; Pollard et al., 2012; Walter et al., 2012). B cell depletion strategies using CD22 antibodies are also being pursued for treatment of a number of autoimmune diseases (Dunussi-Joannopoulos et al., 2005; Leonard & Goldenberg, 2007; Fiorina et al., 2008). In a quite different application, several groups have used anti-siglec antibodies for delivery of antigens to antigen-presenting cells. In particular, antigens conjugated to anti-sialoadhesin (Siglec-1) and anti-Siglec-H are efficiently delivered to macrophages and plasmacytoid DCs, respectively, resulting in modulation of antigen-specific immune responses (Backer et al., 2010; Delputte et al., 2011; Loschko et al., 2011).

In recent years, glycan ligands have begun to provide an alternative to antibodies for targeting siglec-expressing cells. This has been stimulated as a result of advances in the generation of siglec ligands with high affinity and selectivity coupled with multivalent platforms to display them (Collins et al., 2006; Zaccai et al., 2007; Blixt et al., 2008; Magesh et al., 2011; Mesch et al., 2012). As observed with antibodies, ligation of Siglec-8 with the polymeric ligand induces apoptosis of eosinophils (Hudson et al., 2009), suggesting that a suitable ligand-bearing nanoparticle might be used to suppress eosinophil mediated inflammation. Efforts to develop siglec decorated nanoparticle platforms that can deliver therapeutic cargo to targeted cells are also bearing fruit (O'Reilly et al., 2008; O'Reilly & Paulson, 2009; Chen et al., 2010, 2012). For example, CD22 ligand decorated liposomal nanoparticles loaded with the chemotherapeutic doxorubicin have recently been shown to prolong survival in a mouse model of human B cell lymphoma (Chen et al., 2010). This is an attractive platform since siglec ligands can be covalently attached to lipids, allowing simple incorporation into liposomes, which are a pharmaceutically proven nanoparticle formulation.

Many of these targeting applications rely on the fact that the siglecs are endocytic receptors that carry the anti-siglec antibody or siglec-ligand conjugate into the cell (Crocker et al., 2007; Crocker & Redelinghuys, 2008). All siglecs studied to date have been found to be endocytic receptors, including sialoadhesin, CD22, CD33, MAG, Siglec-5, Siglec-6, Siglec-7, Siglec-9, Siglec-F, and Siglec-H (Lock et al., 2004; Biedermann et al., 2007; Scott et al., 2008; Winterstein et al., 2008; O'Reilly & Paulson, 2009; Delputte et al., 2011). While CD22 is endocytosed by a clathrin-dependent mechanism and constitutively recycles between the cell surface and endosomes, Siglec-F is endocytosed by a non-clathrin dependent mechanism and traffics directly to lysosomes (Tateno et al., 2007; O'Reilly et al., 2011). Whether this occurs with Siglec-8 is currently under investigation.

The fact that CD22 is a recycling receptor has consequences for targeted delivery of agents to B cells. Glycan ligands are released in the acidic endosome, allowing the free CD22 to recycle to the cell surface, as illustrated in Fig. 3, which depicts targeting of anti-nitrophenol (NP) IgM to B cells via a hetero-bifunctional ligand comprising NP conjugated to a CD22 ligand (O'Reilly et al., 2008). In contrast, anti-CD22 antibody (clone HIB22) is not released in the endosomes and the CD22-antibody complex recycles between cell surface and endosomes (O'Reilly et al., 2011). As a result, the agent targeted via glycan ligands accumulates in the cell (e.g. the anti-NP-IgM), while the anti-CD22 antibody is not released, and instead equilibrates between the cell surface and endosomal compartments with no net accumulation over time (O'Reilly & Paulson, 2009; Chen et al., 2010). Such considerations will have increasing importance as siglecs, including Siglec-8, are evaluated as receptors for

cell-based targeted therapeutics, and suggest that more work should be done to assess the endocytic mechanisms of siglecs.

In considering what diseases might be particularly amenable to a therapeutic agent like one targeting Siglec-8 that induces eosinophil apoptosis and inhibits mast cell degranulation, several disorders deserve consideration, not the least of which is asthma. Of potential relevance to a Siglec-8-based therapeutic is that genetic linkage studies of asthma and related phenotypes have previously implicated chromosome 19q13.33–q13.41, where most siglec genes are located (Ober et al., 1998; Venanzi et al., 2001). Further encouragement in support of an important role of Siglec-8 in asthma comes from a study of the genetic association between sequence variants in Siglec-8 and the diagnosis of asthma, where a significant association with asthma was observed for various single nucleotide polymorphisms among African American, Japanese and Brazilian populations (Gao et al., 2010). Whether targeting multiple cell types via Siglec-8 will turn out to be more efficacious in asthma than targeting just the eosinophil with anti-IL-5 antibodies like mepolizumab or reslizumab, the eosinophil and basophil with anti-IL-5 receptor antibodies like benralizumab or the mast cell and basophil with omalizumab remains to be seen (Bochner & Gleich, 2010; Cook & Bochner, 2010).

Beyond asthma, there are other disorders, many of which are orphan diseases, in which eosinophils and/or mast cells are believed to play an important role. Examples include chronic rhinosinusitis with nasal polyposis, eosinophilic gastrointestinal disorders (eosinophilic esophagitis, gastritis and colitis), anaphylaxis, chronic idiopathic urticaria, eosinophilic cellulitis and fasciitis, chronic eosinophilic pneumonia, Churg-Strauss syndrome, hypereosinophilic syndromes, and hematologic malignancies involving eosinophils and mast cells, the latter including systemic mastocytosis. While glycan-based therapeutic strategies with ligands that bind to both Siglec-8 and Siglec-F may be fruitfully tested in mice (at least for eosinophil targeting given the shared activity of the 6'-sulfo-sLe^x ligand), transgenic models in which Siglec-8 is selectively expressed in eosinophil and/or mast cell compartments, or models where human stem cells are transplanted into immunocompromised mice resulting in maturation and engraftment of human mast cells (Takagi et al., 2012) would be more beneficial for testing mAb-based agents. This could then be followed by testing of anti-eosinophil and mast cell activity in chimpanzees, the only other non-human species to express Siglec-8 (Angata et al., 2004; Cao et al., 2009). Thus, it is fair to say that the regulatory path to the clinic is not straightforward, yet far from insurmountable.

8. Summary and conclusions

Siglec-8, selectively expressed on human eosinophils, mast cells and weakly on basophils, is an intriguing choice for the development of therapies, such as mAbs and glycan-targeting agents including liposomes, for a variety of disorders including asthma, chronic rhinosinusitis, chronic urticaria, hypereosinophilic syndromes, mast cell and eosinophil malignancies and eosinophilic gastrointestinal disorders where excessive, pathologic involvement of eosinophils and mast cells is occurring. Such targeting, depending on the strategies used, could either leverage the known effects of Siglec-8 engagement, namely induction of eosinophil apoptosis and inhibition of mast cell mediator release, or instead could result in depletion of these cells via delivery of toxic payloads via internalization of Siglec-8-binding glycan-coated liposomes or the use of Siglec-8 mAbs that are humanized to maximize antibody-dependent cell-mediated cytotoxicity. Whether engagement of Siglec-8 by naturally occurring lung cell-derived glycan ligands occurs *in vivo* remains to be determined, but data suggests that such materials may indeed exist in the airways. Regardless, the unique patterns of Siglec-8 expression and mechanisms of inhibition

represent a promising and unique opportunity for its exploitation as a novel therapeutic target.

Acknowledgments

The authors thank Jacqueline Schaffer for Figs. 1 and 2 and Mary O'Reilly for Fig. 3. This work was supported by grants AI72265, AI50143 and HL107151 from the National Institutes of Health. Dr. Bochner also received support as a Cosner Scholar in Translational Research from Johns Hopkins University.

Abbreviations

6'-sulfo-sLe^x	6'-sulfated sialyl Lewis X (NeuAcα2-3(6S)Galβ1-4 (Fucα1-3)GlcNAc
CD	cluster of differentiation
Fc	fragment crystalizable region of IgG
Ig	immunoglobulin
IL	interleukin
ITIM	immunoreceptor tyrosine-based inhibitory motif
mAb	monoclonal antibody
mTEC	mouse tracheal epithelial cells
ROS	reactive oxygen species
Siglec-8-Fc	a fusion protein containing two extracellular Siglec-8 domains linked to the human Fc (fragment crystalizable) region of IgG
Siglec-F-Fc	a fusion protein containing two extracellular Siglec-F domains linked to the human Fc (fragment crystalizable) region of IgG

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Fig. 1. Nomenclature and key structural characteristics of human siglecs. Although 15 are shown, Siglec-13 is present in nonhuman primates but not in man. V structures indicate the arginine-containing V-set domains with lectin activity; these are followed by C2-type Ig repeat domains. U-shaped structures for Siglec-XII indicate the mutated V-set domains missing arginine that have lost their lectin activity. Also shown is DAP12, illustrated as a shorter transmembrane structure co-associating with Siglec-13, Siglec-14, and Siglec-15. See key for symbols representing cytoplasmic signaling motifs. Reproduced from von Gunten and Bochner (2008), with permission.

Fig. 2. Nomenclature and key structural characteristics of mouse siglecs. V structures indicate the arginine-containing V-set domains with lectin activity; these are followed by C2-type Ig repeat domains. Also shown is DAP12, illustrated as a shorter transmembrane structure co-associating with Siglec-15 and Siglec-H. Whether Siglec-H, shown as having an arginine-containing V-set domains with lectin activity, can bind sialic acid ligands remains controversial but there is no human counterpart. Note that Siglecs-1–4 are conserved with humans, as is Siglec-15 (compare to Fig. 1). While not true orthologs, the closest functional paralog of Siglec-E is Siglec-9, while Siglec-F resembles Siglec-8 and Siglec-G resembles Siglec-10. Reproduced from von Gunten and Bochner (2008), with permission.

Fig. 3.

CD22 ligand decorated cargo is released in endosomes and accumulates in the cell, while anti-CD22 antibody recycles to the cell surface. Illustrated is the fate of an anti-NP-IgM ('the cargo') targeted to CD22 on B cells using a heterobifunctional ligand comprising the antigen, NP, coupled to a high affinity ligand of CD22. Following ligand driven assembly of the anti-NP IgM:CD22 complex on the surface of the cell, endocytosis results in release of the anti-NP IgM in the acidic endosomal compartments. CD22 then goes back to the cell surface to capture another load of cargo, resulting in the accumulation of the glycan-based cargo (anti-NP IgM) inside the cell. In contrast, an anti-CD22 antibody (clone HIB22) is not released from CD22 in the endosomes, resulting in the complex of CD22 and the anti-CD22 antibody recycling between the cell surface and the endosomes with no accumulation of the antibody. By permission of Mary O'Reilly.

Table 1

Patterns of expression of siglecs on human cells.

Cell	Siglec														
	1	2	3	4	5	6	7	8	9	10	11	XII	14	15	
B cell		+			±	±				+			±		
Basophil		±	+		+	+		±					+		
CD4 T cell	None														
CD8 T cell							+								
CD34+ cell			+		+			+		+			+		
Dendritic cell			+				+		+	+					
Eosinophil							+	+		±					
Epithelial cell												+			
Macrophage		+		+		+					+		+	+	
Mast cell				+		+	+		+				+		
Microglial cell											+				
Monocyte				+		+		+		+	+	+	+	+	
Neutrophil			+		+			+	+				+		
NK cell				+			+		±	+					
Oligodendrocyte				+											
Placental trophoblast						+									
Schwann cell				+											

Modified from von Gunten and Bochner (2008), with permission.

Table 2

Comparison of Siglec-8 and Siglec-F surface expression on primary cells and cell lines as determined by flow cytometry.

Cell type	Siglec-8	Siglec-F
Primary human cells		
Alveolar macrophages	–	++
B cells	–	–
Basophils	+	ND
Eosinophils	+++	+++
Eosinophils grown from CD34+ cells	+++	+++
Stem cells	–	–
Mast cells	++	–
Mast cells grown from progenitor cells	++	–
Monocytes	–	–
Neutrophils	–	±
NK cells	–	–
T cells	–	±
Primary baboon blood cells		
All leukocytes including eosinophils	–	ND
Primary cynomolgus monkey blood cells		
All leukocytes including eosinophils	–	ND
Primary rhesus monkey blood cells		
All leukocytes including eosinophils	–	ND
Primary dog blood cells		
All leukocytes including eosinophils	–	ND
Cell lines		
Human eosinophil-like		
HL60, AML14, AML14.3D10, EOL-1	–*	ND
Human mast cell-like		
HMC-1.2, LAD2, LUVA	++	ND
HMC-1.1, KU812	–	ND
Dog C2 mastocytoma	–	ND

–: not expressed.

±: Expressed weakly or only under inflammatory conditions.

+, ++, +++: low, moderate or high expression.

ND: not determined.

* Siglec-8 mRNA expression was detected in eosinophil-committed AML14 and AML14.3D10 cells but no protein expression was detected.

Data are from Kikly et al. (2000), Yokoi et al. (2006), Stevens et al. (2007), Tateno et al. (2007), Zhang et al. (2007), Dyer et al. (2008), Hudson et al. (2011), Salicru et al. (2012).

Table 3

Glycan ligand specificities of selected human and murine siglecs.

Glycan ligand	Siglec specificity [†]					
	hCD22	mCD22	hSig-8	mSig-F	Sig-7	hSig-9
•	○	—	○	○	○
•	●	—	—	○	○
●	○	—	—	—	*
—	—	—	○	○	○
—	—	—	—	○	○
—	—	●	●	*	*
—	—	●	●	•	—
—	—	—	○	*	*
—	—	—	○	•	●

Glycan ligand	Siglec specificity [‡]				
	hCD22	mCD22	hSig-8	mSig-F	Sig-7
—	—	—	—	●
					—

Key: ● Gal ♦ Fuc ■ GalNAc ■ GlcNAc ♦ NeuAc ○ NeuGc

[‡] Glycan structures represent terminal sequences of glycans of mammalian glycoproteins and glycolipids that interact with siglecs. Relative preferences of each siglec are compiled primarily from glycan array data from the Consortium for Functional Glycomics (<http://www.functionalglycomics.org>). See also reviews on this subject. Relative strengths of interactions are expressed as strong (large filled circle), moderate (small filled circle), weak (small open circle) or not detectable (—). The * symbol indicates not tested.