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Role of Siglecs and Related Glycan-Binding Proteins in Immune Responses and Immunoregulation

Bruce S. Bochner, MD¹ and Nives Zimmermann, MD²

¹Department of Medicine, Division of Allergy-Immunology, Northwestern University Feinberg School of Medicine, Chicago, IL 60611

²Department of Pediatrics, Division of Allergy and Immunology, Children's Hospital Medical Center and Department of Pathology, University of Cincinnati College of Medicine, Cincinnati, OH 45229

Abstract

Eukaryotic cells and extracellular material are heavily decorated by various glycans, yet the understanding of the structure and function of these moieties lags behind our understanding of nucleic acids, lipids and proteins. Recent years have seen a tremendous acceleration of understanding in the field of glycobiology, revealing many intricacies and functional contributions that were previously poorly understood or even unrecognized. This review highlights several topics relevant to glycoimmunology, where mammalian and pathogen-derived glycans displayed on glycoproteins and other scaffolds are recognized by specific glycan-binding proteins (GBPs), leading to a variety of pro- and anti-inflammatory cellular responses. The focus for this review is mainly on two families of GBPs, siglecs and selectins, that are involved in multiple steps of the immune response, including distinguishing pathogens from self, cell trafficking to sites of inflammation, fine-tuning of immune responses leading to activation or tolerance, and regulation of cell survival. Importantly for the clinician, accelerated rates of discovery in the field of glycoimmunology are being translated into innovative medicinal approaches that harness the interaction of glycans and GBPs to the benefit of the host, and may soon lead to novel diagnostics and therapeutics.

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Correspondence to: Bruce S. Bochner, MD, Samuel M. Feinberg Professor of Medicine, Division of Allergy-Immunology, 240 E. Huron St., Room M-306, Chicago, IL 60611, Phone (312) 503-0068, FAX (312) 503-0078, bruce.bochner@northwestern.edu.

Conflict of interest statement

Dr. Bochner is a co-inventor on existing and pending Siglec-8-related patents and may be entitled to a share of future royalties received by Johns Hopkins University on the potential sales of such products. Dr. Bochner is also a co-founder of 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 and Northwestern University in accordance with their conflict of interest policies. Dr. Bochner has current or recent consulting or scientific advisory board arrangements with Sanofi-Aventis, Merck, GlaxoSmithKline, TEVA, and Allakos; and owns stock in Allakos and Glycomimetics, Inc.

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Keywords

Siglec; selectin; dectin; glycan; glycan-binding proteins; sialic acid; treatment; glycobiology

Introduction

This review will focus on glycans, a term used to describe substances involving sugar (saccharide) moieties linked together, either alone as chains or existing as glycoconjugates (glycolipids and glycoproteins); and the lectins that bind glycans, so-called glycan-binding proteins (GBPs). Also included will be a discussion of how glycobiology, meaning the study of how glycan synthesis, via glycosyltransferase enzymes, and the resulting structures of each glycoconjugate, contributes to cell biology, with a focus on the field of immunology (glycoimmunology). There are many intersections of glycobiology and immunology because glycans are involved in a broad array of responses, from providing structural components in cell walls and the extracellular matrix, to functions in secreted forms (e.g., mucins), to cell signaling, trafficking and adhesion. All eukaryotic cells are covered by glycans in the form of glycoproteins and glycolipids, and they significantly affect function, yet we routinely focus mainly on the proteins, ignoring the “glyco” component. Compared with nucleic acids and proteins, glycobiology can be orders of magnitude more complex, resulting in this field being relatively understudied, leading to severe compromise in our understanding of glycan structure and function. Thus, a deeper understanding of glycobiology in general, including how it relates to immunology, has become a top research priority (for instance, see <http://www.ncbi.nlm.nih.gov/books/NBK109958/>).

Among the best-known glycan structures are the ABO blood groups on the surface of red blood cells. The glycans are added to the end of polysaccharides on proteins and lipids with a fucosyltransferase generating the H antigen in individuals of type O blood group, and an additional N-acetylgalactosamine (GalNAc) or galactose (Gal) is added in A or B-type individuals, respectively. The two different glycosyltransferases responsible for addition of GalNAc or Gal are encoded by different alleles at a single genetic locus, with one essential amino acid difference between the two transferases. In O-type individuals, null alleles are present. All individuals are exposed to the A, B and H structures, mostly in food substances, but are tolerized to the one displayed on their own cells, thus generating antibodies to the other (please note that A and B individuals are tolerized to H structure as well). This forms the foundation of transfusion and transplantation medicine. Importantly, these complex carbohydrates are also expressed on epithelium, platelets, vascular endothelium, and secreted molecules such as von Willebrand factor (vWF). Thus, the ABO group affects more than transfusion and transplantation medicine, including propensity for thrombosis, infections and malignancy. For instance, blood group O individuals have a lower risk of deep venous thrombosis/pulmonary embolism due, at least in part, to lower plasma levels of vWF because blood group glycans influence the excretion of vWF. This one example highlights the far reach of understanding glycan biology in human health and disease.

Why is our understanding of glycomics (all sugars of a cell or tissue) lagging behind genomics and proteomics? The main reasons for this are the structural complexity of glycans

and glycoconjugates, the greater difficulty in determining their sequence (often requiring mass spectrometry, compared with proteins whose sequence can be determined directly or derived from coding nucleic acids), and the fact that glycan biosynthesis cannot be automatically predicted from a template. However, recent years have seen great advances, and this review will highlight developments in glycobiology and glycoimmunology that have improved our understanding of the immune system that offer the potential to impact patient care.

Glycan-binding proteins

GBPs belong to two major groups: lectins, most of which are members of families with carbohydrate-recognition domains, and glycosaminoglycan (GAG)-binding proteins, which bind mostly sulfated GAGs. This review will focus mainly on siglecs, which are I-type (immunoglobulin superfamily)-type lectins, and selectins, a subset of the C-type (calcium-dependent) lectin family, which collectively function in the immune system in processes such as pathogen recognition and cell adhesion, activation, signaling and death (Figure 1). The reader is referred to additional reviews that cover aspects of GBPs that are beyond the scope of this report, including discussions of other important I-type and C-type lectins as well as galectins.^{1–4}

I-type lectins contain at least one immunoglobulin-like fold, with the group most well characterized being the siglec (sialic acid-binding, immunoglobulin-like lectin) family.^{5–13} Members of this family bind lectins with terminal sialic acids, 9-carbon sugars with N-acetylneuraminic acid (NeuAc) being the most common form in mammalian glycoconjugates. While other GBPs can bind sialic acid-containing glycans, siglecs have great specificity for, and require, sialic acids with which they form extensive molecular interactions. The surface of cells is richly decorated with sialic acid-containing glycans that often “mask” surface siglecs on the same cell because they can attach to adjacent siglecs, a process referred to as “*cis*” binding.⁸ This phenomenon is actively regulated *in vivo* (e.g. by sialidase [also known as neuraminidase] action during immune and inflammatory responses, and by grouping of molecules in lipid raft-like structures on surface membranes). Most individual siglecs show restricted patterns of expression on specific cell types, such as Siglec-8 on eosinophils, mast cells and basophils (Figure 2). This makes them useful cell surface markers, and reflects cell-type specific functions mediated by these siglecs. Most siglecs have cytoplasmic signaling motifs, especially immunoreceptor tyrosine-based inhibition motifs (ITIMs) that transmit inhibitory functions, and immunoreceptor tyrosine-based switch motifs (ITSMs) that can function in inhibitory or activating capacities. A few siglecs co-associate with other cell surface proteins that contain immunoreceptor tyrosine-based activation motifs (ITAMs) that also result in cell activation. Examples of such functions specifically related to immune responses will be elaborated on below.

Another example of important immune-related GBPs are cell adhesion molecules. Adhesion molecules are necessary during all processes of inflammation. One of the early steps in this process is for circulating cells to marginate to the periphery of the intravascular spaces despite the shear forces associated with blood flow. This results in leukocyte tethering and rolling on the endothelium, and members of another GBP family, the selectins, mediate

these processes by binding to specific glycan counter-ligands (Figures 1 and 3). C-type lectins include selectins but also molecules such as dectins and DC-SIGN (Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin or CD209), which are involved in pathogen recognition by myeloid cells. For general information about C-type lectins, please see the preceding article in this issue of the *Journal* by Dr. Ronald Schnaar, while examples of their role in immune function will be expanded on below. Additional online resources, including the Essentials of Glycobiology textbook (<http://www.ncbi.nlm.nih.gov/books/NBK1918/>) and the Consortium for Functional Glycomics website (<http://www.functionalglycomics.org>) provide useful information for those interested in the field.

Expression patterns, ligands and cellular functions of selected siglecs

By way of illustration, this section will describe a subset of highly homologous siglecs, Siglec-7, Siglec-8 and Siglec-9, including their patterns of expression and function, and contrast them with Siglec-10. Siglec-7, Siglec-8 and Siglec-9 have three extracellular immunoglobulin domains (the one most membrane distal possessing the sialic acid-binding lectin function), and intracellularly contain a membrane-proximal ITIM domain and a membrane distal ITSM domain with similar evolutionary ancestry (Figure 2). Siglec-7 is more broadly expressed than most siglecs, while Siglec-8 and Siglec-9 are found on completely non-overlapping cell subsets.^{8, 9, 13} Siglec-7 recognizes α 2-8-linked sialosides as well as a range of viral and bacterial glycoproteins and some gangliosides. In comparison, Siglec-8 (and its murine counterpart Siglec-F) and Siglec-9 (and its murine counterpart Siglec-E) recognize similar, but distinct sulfated sialoside structures such as 6'-sulfo-sialyl Lewis X and 6-sulfo-sialyl Lewis X, respectively.^{14, 15}

Siglec-7 has no obvious mouse counterpart. In contrast, both mouse Siglec-F and human Siglec-8 are functionally convergent paralogs, meaning that they have evolved separately but have similar patterns of cell expression, ligands and function. Siglec-F contains four extracellular domains instead of three for Siglec-8. Human Siglec-9 and mouse Siglec-E are true orthologs.^{16–18} Siglec-10, expressed mainly by B cells and a few others, is the one remaining human siglec with a mouse ortholog, namely Siglec-G, and binds both shared and unique ligands compared to other siglecs (Figure 2). Like Siglec-F, Siglec-10 and Siglec-G differ from Siglec-7, Siglec-8 and Siglec-9 in that they contain four extracellular immunoglobulin domains, but Siglec-10 and Siglec-G contain an additional intracellular membrane-proximal growth factor receptor-bound protein 2 (Grb2) binding domain besides the ITIM and ITSM domains. Siglec-10 and Siglec-G biology is covered in greater depth in a later section.

Due to their ITIM-containing cytoplasmic regions, siglec engagement by extracellular glycan ligands initiates cellular signaling that inhibits immune cell activation via binding and activation of phosphatases, such as Src homology region 2 domain-containing phosphatase-1 (SHP-1) and Src-homology 2-containing inositol 5' phosphatase (SHIP). However, siglecs also have activating functions and these involve distinct signaling pathways. For instance, Siglec-8 induces eosinophil death via two distinct pathways, depending on whether the cells are in a resting or activated state.¹⁹ Engagement of Siglec-8

leads to reactive oxygen species (ROS) production by the cells. In resting cells, this leads to ROS- and caspase-dependent apoptosis.^{20, 21} In contrast, in activated eosinophils in which IL-5 exposure has activated the mitogen-activated protein kinase kinase 1- extracellular signal-regulated kinase 1/2 (MEK1-ERK1/2) pathway, ROS further enhances activation of this pathway leading to a biochemically and morphologically distinct mode of caspase-independent cell death.^{19, 22} Similarly, Siglec-9-induced neutrophil cell death is caspase and ROS-dependent in resting cells but only ROS-dependent in activated cells.²³ However, Siglec-9 and Siglec-E also have ITIM/SHP-1/inhibitory signaling related functions in that both are constitutively associated with SHP-1^{24, 25} and Siglec-E-deficient mice exhibit enhanced neutrophil recruitment in endotoxin-induced acute pulmonary inflammation.²⁵ Intriguingly, Siglec-8 signaling and function in a different cell type, namely mast cells, is distinct from that in eosinophils and involves inhibition of mast cell FcεRI-mediated activation and function rather than cell death.²⁶ Also different is signaling via Siglec-F, where mouse eosinophil apoptosis is caspase-dependent but independent of ROS and SHP-1, even in cytokine-activated cells.²⁷ While engaging Siglec-7 classically leads to ITIM-mediated inhibitory signaling pathways²⁸ and dampens immune responses in NK cells and T cells (discussed in more detail below), a monoclonal antibody that crosslinks Siglec-7 induces production of pro-inflammatory cytokines (IL-1α, IL-6, TNF-α) in monocytes.²⁹ Thus, siglecs engage multiple signaling pathways depending on the context, and the critical decision points for this dichotomy are unclear and require further study.

Like Siglec-8, Siglec-F is expressed on mouse eosinophils, but unlike Siglec-8, Siglec-F is prominently expressed on mouse alveolar macrophages.^{16, 30} Engagement of Siglec-8/-F or Siglec-9 with antibodies and/or artificial ligands *in vitro* cause eosinophil and neutrophil death, respectively (Figure 1).^{23, 30–32} At least for Siglec-F, generation of these ligands *in vivo* requires the specific enzyme ST3Gal-III, an α2,3 sialyltransferase, and mice deficient in this enzyme lack lung ligands for Siglec-F.³³ In contrast, the presumed sulfotransferase required to generate the sulfation in 6'-sulfo-sialyl Lewis X, namely keratan sulfate galactose 6-O-sulfotransferase, is not required for generation of Siglec-F ligands, raising the possibility that endogenous ligands may not require sulfation to bind to Siglec-F.³⁴ The exact identity of these sialoside-carrying glycoprotein ligands for Siglec-F in the airways of mice and humans are actively under study, but at least for Siglec-F appear to include mucins such as Muc5b.^{34, 35}

Expression patterns, ligands and cellular functions of selectins

Each of the three selectins possesses N-terminal extracellular C-type lectin domains, and differ from siglecs in several other ways. Selectins contain an EGF-like domain and 3 to 9 consensus-repeat domains in their extracellular structures instead of immunoglobulin-like domains, and they have no intracellular ITIM or ITSM signaling domains (Figure 3).^{36, 37} Like siglecs, L-selectin is constitutively expressed by leukocytes, but the other two selectins are only expressed upon activation of vascular endothelium (P-selectin is also on activated platelets), where they are ideally situated to influence leukocyte trafficking. Although all leukocytes express selectins and/or selectin ligands, one potential mechanism for preferential leukocyte recruitment during inflammation involves the levels of surface expression of these selectins and selectin ligands, which differ among various leukocytes,

types of vasculature (e.g., postcapillary venules versus high endothelial venules found in lymph nodes) and inflammatory conditions (Figure 3). For example, neutrophils and skin-homing T cells have high levels of glycoprotein ligands for E-selectin compared to eosinophils, so endothelial expression of E-selectin during inflammation tends to favor neutrophil and skin-homing T cell accumulation.³⁸ L-selectin is important for leukocyte migration into many locations, including lymph nodes and secondary lymphoid tissues. P-selectin is preformed inside platelets and vascular endothelium and can be rapidly translocated to the cell surface following cell activation. While selectin ligands can share some basic compositional carbohydrate, such as sialyl Lewis X and related structures, each has its own preferred set of glycan ligands (Figure 3).³⁶

Glycoimmunology involving glycan recognition

Pathogens

While the vast majority of antigens are proteins and lipids, there are examples where immune responses such as antibody production are made against carbohydrates. Most are of the IgM isotype, but some are made as IgG, a prime example being the complement-fixing IgG response to the α -galactose epitope (Gal- α 1,3Gal) found on the vasculature of xenotransplanted organs that results in hyperacute solid organ rejection.³⁹ Production of IgE antibody to this glycan, leading to anaphylaxis during infusion of biologicals glycosylated with this epitope, has also been reported⁴⁰ (and see the accompanying review in this same issue of the *Journal* by Dr. Thomas Platts-Mills).

A different example of IgG antibody responses to glycans includes those displayed on the surface of bacteria. One that should be particularly familiar to physicians screening for humoral responses to glycans involves measurement of IgG antibody production to *Streptococcus pneumonia* serotype-specific polysaccharides following vaccination with polyvalent pneumococcal vaccines.⁴¹ While such antibody responses have a tendency to be of the IgG2 subclass, it is clear that such IgG anti-glycan antibodies are by no means restricted to this subclass.⁴² It is not surprising, therefore, that recent vaccine development efforts are beginning to include strategies stimulating recognition of pathogen-specific glycopeptides.⁴³ Examples of such approaches include attempts to immunize subjects to glycans uniquely displayed on helminths such as *Schistosoma mansoni*⁴⁴ and on viruses including HIV.^{45, 46}

Another important illustration of glycan recognition involves viral and anti-viral responses, such as during influenza infection.⁴⁷ Influenza hemagglutinin (HA, of which there are 18 subtypes for influenza A) is a glycoprotein found on the surface of influenza viruses. It is responsible for binding of pathogenic strains to specific sialic acids on cells of the respiratory tract (Figure 1). In addition, each strain of influenza contains its own type of viral neuraminidase (NA). These neuraminidases are required for viral replication and allow the virus to be released from the infected cell. Each strain of influenza virus is characterized by the type of HA and NA that it carries; e.g., H1N1, and each year's influenza vaccine generates neutralizing human antiviral antibodies that predominantly target the HA glycoproteins. Given the importance of NA in influenza replication, neuraminidase

inhibitors have been developed as anti-viral drugs (e.g., oseltamivir and others) that work by blocking viral NA function, preventing viral budding from the infected host cell.

HIV virus is another infectious agent for which glycan recognition via siglecs may play an important role in disease pathogenesis. Sialoadhesin (Siglec-1, CD169) binds HIV gp120 (indeed, half of the mass of gp120 is carbohydrate) in a sialic acid-dependent manner and promotes leukocyte infection while sialoadhesin surface expression on infected monocytes correlates with viral load.^{48, 49} HIV can also incorporate α 2,3-linked sialic acids in the form of glycosphingolipids into the viral particles.⁵⁰ B cell exhaustion seen in HIV infection can be reversed in these cells by molecularly down-regulating Siglec-6 expression.⁵¹ Finally, by increasing type I interferon production, targeted inactivation of Siglec-G protects mice against other viruses besides HIV including lethal RNA virus infection.⁵²

Siglecs, by virtue of their inhibitory signaling pathways and shared ability to recognize a wide range of configurations of sialic acids, are considered an important part of mammalian innate immune “self” recognition. Unfortunately, pathogens have evolved to play this game too, and as a result some have developed the ability, via cell surface glycocalyx mimicry, to display sialic acids that are mistakenly seen as self.⁵³ For better or for worse, certain siglecs, especially Siglec-7 and Siglec-9, bind specific sialylated bacterial glycans, initiating inhibitory responses in those leukocytes that express these siglecs and dampening their anti-pathogen responses, allowing certain organisms to evade innate immune responses (Figure 2). Such pathogens include group B *Streptococcus*, *Pseudomonas*, *Campylobacter*, *Neisseria* and others.^{54, 55} Other inhibitory siglecs, such as Siglec-5, can co-exist on the same cell surface with Siglec-14, the latter non-covalently associating with activating receptors, such as DAP12, such that engagement facilitates, rather than dampens, innate immune responses. Indeed, a homozygous deficiency in Siglec-14 protects those with chronic obstructive lung disease from exacerbations, but makes their neutrophils more susceptible to Siglec-5-mediated subversion by group B *Streptococcus*.^{56, 57} One final important example of siglec involvement in innate immune responses involves extensive inhibition of toll-like receptor (TLR) function by their direct interaction with several siglecs. These suppressive interactions can be abrogated by elimination of Siglec-E, resulting in augmented dendritic cell responses *in vitro*.⁵⁸ Taken together, it becomes clear that there are situations where glycans on pathogens and other surfaces are recognized for beneficial protective immune responses, as well as examples whereby the pathogen’s glycome is used to undermine a protective response by engaging inhibitory receptors on immune cells.

Immune deficiencies related to GBPs

Given the importance of glycan recognition in host defense, it is not surprising that defects in some of these pathways lead to immune deficiencies. Leukocyte adhesion deficiency (LAD) type 2 is a rare congenital disorder of glycosylation (CDG) also called LAD type 2, or CDG-IIc. It is one of the few types of GDG that results in immune defects (see <http://www.ncbi.nlm.nih.gov/books/NBK1939/>). Patients with this disorder have short stature, mental retardation, Bombay (hh) blood type (due to a deficiency of the red blood cell H antigen, mentioned above in the introduction) and impaired leukocyte trafficking to sites of inflammation.⁵⁹ The disease is due to a defect in a golgi-based GDP-fucose transporter gene

SLC35C1/FUCT1 that is required not only for generation of the H antigen on red blood cells but also for generation of glycans important for selectin binding, including sialyl Lewis X. While not as severe as leukocyte adhesion deficiency type 1 (a lack of $\beta 2$ integrin expression on all leukocytes), both can present with recurrent pneumonias, periodontitis, otitis, impaired pus formation, blood neutrophilia and tissue neutropenia.⁶⁰

Dectin-1, encoded by the *CLEC7A* gene, is a C-type lectin with an extracellular domain that functions as a pattern-recognition receptor, binding to $\beta 1,3$ -linked and $\beta 1,6$ -linked glucans (polysaccharides of glycosidically-linked D-glucose) produced by fungi including *Saccharomyces* and *Candida*. Dectin-1 is expressed by phagocytic cells and B cells, and possesses a cytoplasmic domain with an ITAM such that binding of ligand induces cell activation. There was a recent report of a family in which women with onychomycosis or chronic vulvovaginal candidiasis were found to have a mutation in Dectin-1 resulting in decreased levels of surface expression and function, although fungal phagocytosis and killing were normal.⁶¹ In a related condition, a premature termination codon in the gene *CARD9*, encoding the caspase recruitment domain-containing protein 9, was identified in relatives with chronic mucocutaneous candidiasis and lethal invasive infections. Functional studies suggest that this mutation impairs Dectin-1 signaling.⁶²

Effect of IgG glycosylation on its function

The immunoglobulin molecules are glycosylated with distinct moieties that affect their stability, distribution and function.⁶³ Described in this section are a few examples of such functional effects. Intravenous immunoglobulin (IVIG) is widely used along with subcutaneous delivery for replacement therapy in immunodeficient individuals, but is given at higher doses for immunosuppressive effects in autoimmune and inflammatory diseases. The mechanism of the paradox, wherein IgG has both a pathogenic, pro-inflammatory role and yet can be used for treatment of certain autoimmune diseases, has been extensively studied, yet is still incompletely understood. The IgG Fc fragment is critical for IgG to exert its pro-inflammatory functions, primarily via the activation of innate immunity by the complement system and innate cell activation via Fc γ Rs. Importantly, co-expression of activating and inhibitory Fc γ Rs determines the strength of effector responses following binding of IgG by setting the activation threshold. Affecting this balance is a possible way for IgG to have both pro- and anti-inflammatory properties.

IVIG may counteract IgG-mediated autoimmunity via multiple independent and non-mutually exclusive mechanisms, including one that involves sialylation of the IgG molecule.^{64, 65} Multiple studies support the notion that the Fc portion of the IgG molecule is sufficient for IVIG's anti-inflammatory properties in mouse models, in patients with autoimmune disease such as idiopathic thrombocytopenic purpura.⁶⁶ The effectiveness of IVIG is dependent on the inhibitory receptor, Fc γ RIIB.⁶⁷ Recently, studies have uncovered a critical role for glycosylation, specifically terminal sialic acids,⁶⁸ on the Fc fragment of IgG in IVIG anti-inflammatory function. While siglecs, which are known to bind terminal sialic acids, were a natural first suspect to study as the receptor for sialic acid-mediated IVIG effect, studies have instead shown a critical role for the C-type lectin and pathogen-recognition receptor DC-SIGN (mouse ortholog is SIGNR1).⁶⁹ Specifically, DC-SIGN

activation is thought to then lead to upregulation of inhibitory Fc γ RIIB on inflammatory cells and in turn decreased inflammation. Thus, if this mechanism proves to be critical for IVIG effectiveness, one can envision replacing IVIG, which is a pooled blood product, with synthetic products enriched with terminal sialic acids. With advances in the glycobiology field, synthetic glycans or glycoconjugates may provide a new line of therapeutics for autoimmune and inflammatory disorders (discussed further below).

Another example of glycans influencing IgG biology involves antibody-dependent cellular cytotoxicity (ADCC), the process by which targets (such as cells) coated with IgG antibody are killed by cells such as NK cells, macrophages and neutrophils expressing the IgG receptor Fc γ RIII (CD16). These Fc γ Rs are engaged much more effectively if the IgG Fc region lacks fucose. Such forms of IgG are called afucosylated antibodies. Several methods have been developed to eliminate fucose on therapeutic antibodies, including production of the antibodies in cell lines lacking α 1,6-fucosyltransferase.^{70, 71} There are numerous examples of afucosylated therapeutic antibodies being developed that are designed to deplete cells, including those targeting cancers (e.g., CD19 [MEDI-551], CD20 [afucosylated rituximab], and afucosylated HerceptinTM [trastuzumab]) as well as an antibody to the IL-5 receptor on eosinophils (benralizumab).⁷²

Cancer immunology

A number of primary tumors express glycan ligands for Siglec-7 and Siglec-9, and expression, or lack thereof, of these ligands correlates with cytotoxicity of NK cells towards susceptible versus resistant tumors.⁷³ Furthermore, when lipophilic glycopolymers containing Siglec-7 ligands were introduced into the outer cell membranes of tumor cells, this resulted in hypersialylation of the tumor cell glycocalyx, enhanced engagement of Siglec-7, augmented inhibitory signaling, and protection from NK cell-dependent killing.⁷⁴ The implication here is that cancers expressing high levels of siglec ligands may evade NK cell killing, and that treatments that reduce levels of sialic acid on the surface of cancer cells (or blockade of Siglec-7) might make it easier for the tumors to be killed. In addition, an augmented tumor glycocalyx was shown to enhance integrin clustering and activation, an effect that might enhance metastatic potential.⁷⁵ Finally, malignant cells often express abnormal glycans and glycoproteins, such as the transmembrane mucin MUC-1 and others, that could be exploited as biomarkers and therapeutic targets.⁷⁶

Allergic diseases

Targeting of siglecs has been utilized to reduce eosinophil and/or mast cell numbers and function. Incubation of eosinophils with antibodies or multimeric glycan ligands for Siglec-8 cause eosinophil death *in vitro*,^{19, 20, 22, 32} and exposure of mast cells to these same antibodies inhibit Fc ϵ RI-dependent activation.²⁶ Antibodies to Siglec-7 have a similar inhibitory effect on mast cells and basophils.⁷⁷ Administration of antibodies to Siglec-F, the functional mouse paralog of Siglec-8, in mouse models of eosinophilia, and allergic lung and gastrointestinal eosinophilia nearly normalizes blood and tissue eosinophil numbers and attenuates tissue remodeling.^{78–81} Mice deficient in Siglec-F display exaggerated allergic eosinophilic pulmonary inflammation.^{82, 83} Like Siglec-F deficient mice, ST3Gal-III deficient mice deficient in sialylated Siglec-F ligands demonstrate an exaggerated

eosinophilic pulmonary inflammatory response following allergen sensitization and airway challenge.^{15, 84} While the natural ligands for Siglec-8 and Siglec-F are still being defined, preliminary data implicate mucins, especially Muc5b.^{34, 85}

Autoimmunity and inflammation

The fact that siglecs provide inhibitory signaling (Figure 1) has led to the theory that a defect in this negative regulatory pathway could result in autoimmunity and/or enhanced inflammation. A compelling example in support of this hypothesis comes from biology related to Siglec-G and its human ortholog Siglec-10 expressed on B cells, dendritic cells and others (Figure 2).^{86, 87} Mice deficient in Siglec-G show a variety of abnormalities, including enhanced inflammatory responses to danger-associated molecular patterns (DAMPs, Figure 1), such as high mobility group protein 1,⁸⁸ markedly enhanced B1 B cell numbers and IgM production,⁸⁹ augmented arthritis and lupus-like responses,⁹⁰ and worsen graft versus host disease responses.⁹¹ The B cell expansion is even more exaggerated in mice deficient in two siglecs, Siglec-G and CD22, and these mice also show evidence of autoimmunity.⁹² Physiologic inhibitory signaling via Siglec-10 and Siglec-G occurs as a result of engagement of sialic acid residues on CD24, a glycosyl-phosphatidylinositol-linked protein on the surface of B cells and granulocytes.⁸⁸ In addition to CD24 as a ligand, some antigen-activated human T cells suppress other T cells in a process mediated by soluble CD52 that binds to Siglec-10 and impairs T cell receptor signaling and activation.⁹³ Regardless of physiologic ligand involved, inhibitory signaling via Siglec-G and Siglec-10 appears to be an important mechanism influencing B cell and T cell activation and proliferation.

Other siglecs play important roles in inflammation. Studies of neutrophil recruitment to the lung in a mouse model of sepsis using mice deficient in Siglec-E confirmed its role as a negative regulator of neutrophil migration and function via control of ROS production.²⁵ Studies involving responses to microbial-derived TLR ligands have shown that removal of sialic acids *in vivo* allow more effective responses during infection.⁵⁸ One additional aspect of glycans in autoimmunity and inflammation deserves mentioning, and this is the possible utility of anti-glycan antibodies as biomarkers in disease. IgG autoantibodies to sulfated glycans were particularly prevalent in the sera of patients with scleroderma and pulmonary hypertension,⁹⁴ and antibodies have been detected to unique glycans in Crohn's disease and multiple sclerosis.^{95, 96}

Therapeutic strategies involving glycoimmunology

Regarding potential therapeutic applications involving sialoside analogue-based cell targeting, several high affinity molecules that selectively recognize specific siglecs, such as for sialoadhesin (Siglec-1) on macrophages, CD22 on B cells, Siglec-7 and Siglec-9 have been synthesized (e.g., compound G35 and compound D24 for Siglec-7 and Siglec-9, respectively [Figure 2], as well as others^{97–99}) and can be deployed on liposomes and other nanoparticles to selectively target subsets of siglec-bearing cells for delivery of toxic payloads or other immunomodulatory agents to immune cell subsets.¹⁰⁰ In a related approach to augment immune responses, liposomes decorated with glycan ligands for sialoadhesin on macrophages undergo endocytosis, and cause vigorous iNKT cell activation

via targeted delivery of an appropriate lipid antigen¹⁰¹ Similarly, targeting mycobacterial lipid antigens to dendritic cells via liposomal nanoparticles coated with Siglec-7 ligands cause marked T cell activation.¹⁰²

The inhibitory function of siglecs is being exploited for suppressing unwanted immune responses, such as autoimmunity, transplantation, allergic diseases and others. Current therapeutic approaches mainly involve the use of immunosuppressive drugs; however, this compromises normal immunity and thus carries risks. Novel methods are being explored that would induce antigen-specific tolerance while preserving protective immunity. One such approach exploits the natural mechanisms of B cell suppression by siglecs: coating T-independent antigens with ligands for Siglec-G and CD22 results in suppressive immune responses including B cell tolerance.¹⁰³ Particles that display both the antigen and glycan ligands for the B cell receptor and CD22 induce apoptosis of antigen-specific B cells.¹⁰⁴ This concept was tested in a model of hemophilia A. Siglec-engaging tolerance-inducing antigenic liposomes (STALs) were delivered to mice deficient in factor VIII, and prevented formation of neutralizing anti-factor VIII antibodies. Further evidence of the utility of targeting siglecs in inducing tolerance was shown when liposomal nanoparticles presenting both antigen and a specific Siglec-G ligand (termed F9) inhibited B cell receptor signaling and tolerance toward T-independent and T-dependent antigens.¹⁰⁵ Future studies will need to establish whether STALs are able to abrogate established memory B cell responses *in vivo*. If so, this approach could be expanded to more indications such as the treatment of autoimmune and other unwanted antibody-mediated disorders including IgE-mediated diseases.

Some commercial preparations of replacement IgG contain naturally occurring autoantibodies to both Siglec-8 and Siglec-9 at concentrations that are sufficient to cause eosinophil and neutrophil apoptosis, respectively, *in vitro*.^{106, 107} Whether this explains some of the anti-inflammatory properties seen following administration of high doses of IgG is not known. Two final glycan-related approaches are being investigated for the treatment of autoimmune and inflammatory conditions. One involves the pan-selectin antagonist GMI-1070 given intravenously that is being explored in early clinical trials for its ability to interrupt vaso-occlusive disease in sickle cell crisis,¹⁰⁸ as was shown in a mouse model.¹⁰⁹ Another involves the use of an enzyme, endoglycosidase S, or EndoS, which removes a number of glycans from IgG, reducing FcγR binding.¹¹⁰ This, in theory, could result in selective de-glycosylation of antibodies *in vivo* as a way to treat patients with antibody-mediated autoimmunity where disease pathophysiology is caused by these IgG-FcγR interactions, such as in systemic lupus erythematosus.^{111, 112} One concern with this strategy is that systemic administration of the EndoS enzyme, a bacterial product, itself might be immunogenic. In summary, multiple novel strategies are being developed to combat immune diseases by taking advantage of our increased understanding of the interface of glycobiology and immunology.

Conclusions

While there is great complexity in glycobiology and glycoimmunology, clear patterns for the role of glycans and GBPs in immune responses are emerging. Glycans are one part of the

immune system's ability to distinguish self from danger; however, pathogens can sometimes use their glycocalyx in order to evade immune recognition. Similarly, cancer cells can adapt their glycome as part of an evolutionary advantage to evade immune reactivity. Glycans and GBPs are part of the regulation of recruitment of immune cells to sites of inflammation, and defects in GBPs or their ligands can lead to immunodeficiencies. The level of immune response or tolerance is regulated in part by glycans and GBPs, and knowledge of this balance is guiding targeted therapy using novel approaches involving glycans, including vaccination. Several tactics exploiting glycoimmunology have already or will soon make their way to the clinic, and it is anticipated that additional therapeutic approaches will emerge as our understanding of the glycome and its function in immune responses expands.

Abbreviations

ADCC	antibody-dependent cellular cytotoxicity
DAMP	danger-associated molecular pattern
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
ERK	extracellular signal-regulated kinase
E-selectin	endothelial-selectin (CD62E)
FcγR	IgG receptors
GAG	glycosaminoglycan
Gal	galactose
Gal-α1	3Gal, the oligosaccharide galactose- α1,3 galactose, also called α-Gal
GalNAc	N-acetylgalactosamine
GBP	glycan-binding protein
Grb2	growth factor receptor-bound protein 2
HA	hemagglutinin
IL	interleukin
ITAM	immunoreceptor tyrosine-based activation motif
ITIM	immunoreceptor tyrosine-based inhibition motif
ITSM	immunoreceptor tyrosine-based switch motif
IVIG	intravenous immunoglobulin
L-selectin	leukocyte-selectin (CD62L)
MEK	mitogen-activated protein kinase kinase
NA	neuraminidase, also called sialidase
NeuAc	N-acetylneuraminic acid, a type of sialic acid
P-selectin	platelet-selectin (CD62P)

ROS	reactive oxygen species
SHIP	Src-homology 2-containing inositol 5' phosphatase
SHP-1	Src homology region 2 domain-containing phosphatase-1
STAL	siglec-engaging tolerance-inducing antigenic liposomes
TLR	toll-like receptor
TNF	tumor necrosis factor
vWF	von Willebrand factor

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Figure 1. Examples of functions of GBPs on immune cells

GBPs are involved in multiple steps of the immune response, including A) pathogen recognition and binding, which for example on monocytes and other cells can lead to productive immunity or help pathogens evade the immune response if inhibitory pathways are engaged; B) rolling and adhesion of leukocytes that are being recruited to sites of inflammation via selectins and their glycan ligands, and whose regulated expression leads to preferential recruitment of immune cell subsets at sites of inflammation; C) inducing cell death immune cells, for instance with eosinophils and neutrophils when Siglec-8 or Siglec-9, respectively, are engaged by endogenous sialoside ligands; and D) fine-tuning of the immune response leading for example to B cell activation or tolerance by influencing signaling thresholds by modulating protein phosphatases.

Siglec (mouse ortholog or paralog)*	Human cell expression	Ligands**	Functions
Siglec-7 (none)	Ba, CD8, DC, E, MC, Mo, NK	α 2-8-linked sialosides; 6-sulfo sialyl Lewis X; sialyl Lewis X, disialyl Lewis A; HIV-1 gp120; compound G35; <i>Campylobacter jejuni</i> and <i>Pseudomonas aeruginosa</i> glycans; gangliosides GT1b, GD3 and DSGb5	Inhibition of basophil and mast cell activation; inhibition of anti-bacterial and anti-cancer cell responses
Siglec-8	Ba, E, MC	6'-sulfo sialyl Lewis X; 6'-sulfo-sLacNAc; mucins; some replacement IgG preparations	Induction of eosinophil cell death; inhibition of mast cell activation
(Siglec-F)
Siglec-9	B, Mac, Mo, N, NK	6-sulfo sialyl Lewis X; prohibitins; vascular adhesion protein-1; compound D24; MUC1 and MUC16; <i>Pseudomonas aeruginosa</i> and group B <i>Streptococcus</i> glycans; some replacement IgG preparations	Inhibition of anti-bacterial and anti-cancer cell responses; neutrophil apoptosis
(Siglec-E)
Siglec-10	B, E, Mo, NK	α 2-3 and α 2-6-linked sialosides; CD24; CD52; compound F9; vascular adhesion protein-1	Control of B cell and T cell activation; control of autoimmune responses; induction of B cell tolerance
(Siglec-G)

Figure 2. Selected examples of human siglecs and their closest mouse counterparts, cellular expression, ligands and function

*The extracellular N-terminal lectin binding domains are shown in blue and the Ig domains in red, while the intracellular ITIM and ITSM domains are shown as red balls and yellow balls, respectively. Siglec-10 and Siglec-G also contain a membrane-proximal Grb2 binding domain shown as red discs. ** Ligands listed include specific glycans or sialoside analogues themselves, glycoproteins or glycolipids decorated with specific glycans, or commercial IgG preparations used clinically that contain detectable levels of anti-siglec autoantibodies. Abbreviations: B lymphocyte; Ba, basophil; B, CD8, CD8+ T cell; DC, dendritic cell; Mac, macrophage; MC, mast cell; Mo, monocyte; N, neutrophil; NK, NK cell; Plac, placental trophoblast; sLacNAc, sialyl N-acetyl-D-lactosamine

Selectin (CD designation)*	Cellular expression	Ligands**	Functions
<p>E-selectin (CD62E)</p>	Activated post capillary venular endothelium	Sialyl Lewis X; ESL-1, α 2-3-linked sialylated glycosphingolipids; MUC16; endoglycan	Leukocyte rolling, adhesion, and extravasation on inflamed post-capillary venules
<p>L-selectin (CD62L)</p>	All leukocytes	6-sulfo sialyl Lewis X; CD34; CD44 isoforms; MadCAM-1; PSGL-1 (CD162); MUC16; endoglycan; heparan sulphate	Leukocyte rolling, adhesion, and extravasation; trafficking of lymphocytes to lymph nodes and other secondary lymphoid organs
<p>P-selectin (CD62P)</p>	Activated platelets and endothelium	6-sulfo sialyl Lewis X; PSGL-1 (CD162); CD44 isoforms; TIM-1 glycoprotein; heparan sulphate	Leukocyte rolling, adhesion, and extravasation; aggregation of activated platelets

Figure 3. Selectin adhesion molecules, and their cellular expression and ligands

* The N-terminal extracellular C-type lectin domains are shown as green crescents, the epidermal growth factor-like domain as a yellow rod, and the complement control protein-like repeat domains as blue spheres. ** Ligands listed include specific glycans or sialoside analogues themselves, glycoproteins or glycolipids decorated with specific glycans.