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The Regulatory Power of Glycans and their Binding Partners in Immunity

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Abstract

Glycans and glycan-binding proteins are central to a properly functioning immune system. Perhaps the best known example of this is the selectin family of surface proteins that are primarily found on leukocytes, and which bind to endothelial glycans near sites of infection or inflammation and enable extravasation into tissues. In the past decade, however, a number of other immune pathways that are dependent on or sensitive to changes in glycan-mediated mechanisms have been revealed. These include antibody function, apoptosis, Th1 versus Th2 skewing, T cell receptor signaling, and MHC class II antigen-presentation. Here, we highlight how regulated changes in protein glycosylation both at the cell surface and on secreted glycoproteins can positively and negatively modulate the immune response.

Keywords

Glycosylation; Glycans; Immune Regulation; Galectin; C-type Lectin; MHCII; Antibody

Host Glycans and their Ligands

Host glycans can take many forms, ranging from glycosaminoglycans (GAGs) such as heparin, chondroitin sulfate, and hyaluronan, to the canonical glycoprotein-associated glycans. Protein glycosylation is the enzymatic process of adding carbohydrates to specific sites on nascent proteins (Figure 1)^{1,2}. Recent genomic studies reveal the presence of carbohydrate recognition domains (CRDs) throughout evolution, suggesting an important role for glycans and their recognition in physiology³. In addition, new techniques in mass spectrometric analysis allow definition of precise glycan compositions, including linkages and other features, providing a deeper understanding of glycan biology^{4,5}. With these advances, protein glycosylation (Figure 1; Box 1) is emerging as an important regulatory mechanism in the immune response. Given the overlapping epitopes found in N- and O-linked glycans, we will focus on these glycans and their binding partners, but O-GlcNAc (N-acetylglucosamine) and other glycans (e.g., GAGs and glycolipids) are also important in immunity as described elsewhere^{6–11}.

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Box 1**N- and O-Linked Protein Glycosylation**

Glycans attached to glycoproteins can be divided into three broad categories: asparagine (N)-linked, serine/threonine (O)-linked, and O-linked N-acetylglucosamine (O-GlcNAc). Many nuclear and cytoplasmic proteins are glycosylated by the addition of a single N-acetylglucosamine (GlcNAc) on serine or threonine residues by O-GlcNAc transferase (OGT), whereas essentially every secreted and integral membrane protein is glycosylated by N- or O-linked glycans within the ER and Golgi apparatus. Classical O-glycans are built *de novo* directly on the protein within the Golgi apparatus, which is initiated by the α -linked addition of a N-acetylgalactosamine (GalNAc) carbohydrate to a serine or threonine residue by the polypeptide GalNAc transferase (PP-GalNAcT) via the available hydroxyl group on the amino acid side chain, hence the name “O-linked”. If left unmodified, the single GalNAc is known as the Tn antigen; however, these structures are usually built up by the sequential action of Golgi-resident enzymes to form mature glycans, which are classified into eight “Cores” (Core 1–8) depending on the carbohydrate(s) proximal to and their linkage with the initiating GalNAc. For example, the addition of a galactose (Gal) residue by β 1,3 linkage turns the Tn antigen into the Core 1 glycan (also known as the T antigen). Further addition of a GlcNAc residue to the Core 1 glycan by β 1,6 linkage creates the Core 2 glycan. The other Core glycans are variations on this theme, with differing carbohydrates and linkages proximal to the initial GalNAc. In all cases, these glycans can be further elongated to highly complex structures carrying a variety of carbohydrates in multiple combinations and glycosidic linkages, including terminal sialic acids, Gal, GalNAc, GlcNAc, fucose (Fuc), and others.

In contrast, N-glycans begin as a relatively large core structure which is initially synthesized as a lipid precursor on the cytoplasmic face of the endoplasmic reticulum (ER) membrane, moved into the ER lumen during synthesis by the ER-localized Flipase, and then added to nascent polypeptides at the available primary amine on asparagine residue side chains within the “N-x-S/T” consensus sequence, hence the name “N-linked”¹⁰¹. Although not all N-xS/T sites are glycosylated, this transfer is catalyzed in the ER by the oligosaccharyltransferase (OST) enzyme complex, which is the target of the often used drug tunicamycin which prevents the addition of N-glycans and causes significant ER stress and the initiation of the unfolded protein response. Within the ER, N-glycans play a role in the quality control of protein folding by mediating interactions with ER-resident chaperones such as calreticulin and calnexin; however, once out of the ER and into the Golgi, the N-glycans on nascent proteins are trimmed to “high mannose” structures dominated by terminal mannoses before being rebuilt into the complex N-glycans common to mammalian glycoproteins. The transition between a high mannose N-glycan and a complex-type N-glycan is the addition of a GlcNAc on one arm of the mannose core by GlcNAcT1 (Mgat1). This transitional glycan structure with a single GlcNAc (which can be elongated with the other carbohydrates) and terminal mannose residues on the other arms is called a “hybrid” N-glycan. Upon subsequent addition of GlcNAc residues, the N-glycans fall into the “complex N-glycan” category, with each additional GlcNAc representing a new glycan “branch”. As these GlcNAc branches are added, it is common to term the resulting N-glycans bi-, tri-, and tetra-antennary, based on how many branches are present. N-glycans typically develop into structures with at least two branches (bi-antennary) and are built up through the sequential action of Golgi enzymes in much the same fashion as O-glycans.

N- and O-linked glycans are found on nearly all membrane and secreted proteins produced by mammalian cells and can carry key immunologic epitopes, such as the canonical blood

group antigens H, A, and B as well as the Lewis blood antigens (e.g., sialyl-Lewis^X) (Figure 1)^{1,2}. Another epitope found within N- and O-linked glycans is N-acetylglucosamine (GlcNAc), which is a disaccharide of galactose (Gal) and GlcNAc that can be present as a single disaccharide unit or in repeated fashion (poly-LacNAc) (Figure 1). LacNAc or poly-LacNAc structures represent the minimal glycan structure required for many of the mammalian β -D-galactoside-binding lectins, galectins¹², which are integral to the regulation of immunity. In addition to epitopes found within the N- and O-linked glycans themselves, modifications such as sialylation and fucosylation (Box 1) can alter the biology of the glycan substantially and are highly regulated (Figure 1). For example, glycans with the LacNAc motif are ligands for galectin-1 (gal-1), but addition of a terminal sialic acid can “cap” the epitope and prevent gal-1 binding¹³. Likewise, lymphocyte development is controlled through fucosylation of the NOTCH pathway (reviewed in [^{14,15}]).

Although glycan function is highly specific to the particular glycoprotein on which the glycan is present, it often relies upon glycan-binding proteins, such as galectins, sialic acid binding Ig-like lectins (siglecs), and C-type lectin receptors (CLRs). The regulation of glycan structures and glycan-binding protein expression, and their complex interplay, are increasingly recognized as crucial features of a properly functioning immune system (Figure 2). Glycan function in the immune system is perhaps best known for its role in selectin binding (reviewed in [^{16–18}]). Selectins are surface-localized members of the CLR family that bind to glycans and mediate leukocyte rolling and ultimately lead to extravasation into inflamed or infected tissues with the assistance of the integrins. As such, the regulation of glycan structures on endothelial and leukocyte surfaces during an inflammatory response is a key factor in the recruitment and homing of immune cells to the affected area.

Here, we highlight how regulated changes in protein glycosylation both at the cell surface and on secreted glycoproteins can positively and negatively modulate the immune response. This is well documented for the glycan-binding proteins selectins and siglecs that control leukocyte homing and self versus non-self discrimination, respectively and has been reviewed elsewhere^{18–24}. New work shows that glycans and their binding partners regulate antibody function, antigen presentation (e.g., MHCII), skewing of T cell responses, immune signaling cascades and innate recognition of microbial products. Like regulation of transcription and protein phosphorylation, the regulation of glycan synthesis and glycan-binding lectin expression is central to the immune response.

Glycosylation acts as a switch between pro- and anti-inflammatory IgG functionality

Antibodies are tetrameric glycoproteins that play a crucial role in directing immune responses. IgG is the major antibody isotype in the circulation and IgA is the dominant form in the mucosa. Each antibody consists of two copies of the heavy chain and two copies of the light chain, which together contain variable antigen specificity-determining (Fab) domains positioned away from a conserved functional (Fc) domain that modulates the cellular response in a way that is dependent on the type of cell-surface Fc receptor proteins engaged by the antibody (e.g. Fc γ Rs for IgG). All IgG Fc domains contain a single, highly conserved, glycosylation site (Asn297) that carries complex N-glycans (Box 1; Figure 1) that are required for Fc receptor binding, probably due to the structural role they play in stabilizing the overall conformation of the region²⁵. Over 30 glycan variations have been detected, but they are typically limited to biantennary structures (Figure 1) with and without terminal sialic acids²⁶. Recent evidence suggests that Fc-localized N-linked glycans directly impact IgG antibody function.

Glycans modulate IgG Fc function, in part, by altering the binding affinity for different Fc γ Rs. IgG containing glycans that lack core fucose have increased affinity for Fc γ RIIIA²⁷, a receptor involved in antibody-dependent cell-mediated cytotoxicity (ADCC)²⁸. IgG containing a bisecting GlcNAc (Figure 1) have increased affinity for Fc γ RIII²⁹, a receptor that mediates neutrophil recruitment³⁰ and which is required for the development of collagen-induced arthritis³¹. Terminal sialylation seems in general to decrease affinity to Fc γ Rs³².

One effective treatment for patients with autoimmune disease is the use of high-dose intravenous immunoglobulin (IVIg), which robustly suppresses the inflammatory response. In 2006, it was discovered that IgG antibodies with Fc domains carrying α 2,6-linked sialylated N-glycans represent the anti-inflammatory portion of the IVIg²⁶. Although sialylated IgG (sIgG) comprises only about 10 % of total IVIg, when enriched, sIgG shows a 100-fold increase in anti-inflammatory activity over IVIg by weight in a mouse model of arthritis²⁶. In parallel experiments, *in vitro* α 2,3-linked sialylation of IgG Fc did not result in anti-inflammatory activity, demonstrating that this effect is specific for the α 2,6 linkage³³.

Although sIgG shows reduced binding affinity (decreased K_a values) to both activating and inhibitory Fc γ Rs²⁶, the anti-inflammatory mechanism of the sIgG fraction of IVIg appears to be mediated through binding to the CLR DC-SIGN (SIGN-R1 in mice) (Figure 2)³⁴. Murine marginal zone macrophages, or human DCs, bind to sIgG and produce IL-33 leading to expansion of IL-4-secreting basophils. Effector macrophages then respond by increasing expression of the inhibitory ITIM-containing Fc receptor Fc γ RIIB, which shifts the response away from the damaging pro-inflammatory cascade (Figure 2). This mechanism was reported to suppress arthritis in one murine model of the disease³⁴, but more recent data suggests other mechanisms through which N-glycans may enable IgG-mediated suppression of the immune response³⁵.

Most Fc γ Rs send activating signals that can synergize with the complement protein C5a and its receptor C5aR to elicit a pro-inflammatory cascade that is relevant to several immune-mediated diseases³⁶. Recent findings reveal that IgG1 immune complexes can suppress C5aR to inhibit inflammation through the coordinated action of both Fc γ RIIB and the CLR dectin-1 (Figure 2)³⁵. More specifically, IgG1 complexes induce Fc γ RIIB and dectin-1 association, leading to a blockade in ERK1/2 phosphorylation downstream of C5aR signaling. In contrast to earlier work^{26,33,34}, this study revealed that the inhibitory IgG1 requires high amounts of galactosylation (gIgG), while the presence of terminal sialic acids appears irrelevant for activity, further demonstrating the power of IgG glycosylation in determining biological functions.

Given these data, it is not surprising that changes in concentration of IgG Fc glycans are reported in patients with autoimmunity and inflammatory disease. For example, rheumatoid arthritis (RA) and Wegener's granulomatosis patients produce lower levels of sIgG compared to normal controls^{37,38}. During remission, these patients show increased amounts of α 2,6-sialylated IgG in the circulation, suggesting that sialylation is dynamically regulated³⁸. Loss of terminal galactose residues (agalactosylation) on IgG1, and subsequent loss of inhibitory gIgG, is also seen in patients suffering from RA and can appear in circulation preceding disease onset^{39,40}, however, the regulatory mechanisms leading to the generation of these IgG glycoforms remains unclear. One study showed that IL-21 is capable of modulating IgG glycosylation from B cells *in vitro*⁴¹. Another group demonstrated the need for an intact promoter region of ST6Gal-1, the enzyme responsible for creating α 2,6 sialyl-linkages, to produce anti-inflammatory IgG during inflammatory progression⁴². Work from a third group suggests that antigen-specific regulation of sIgG

forms under tolerogenic T cell-dependent signaling during allergic inflammation⁴³. Much work remains to fully understand the complex regulatory program required to dynamically manipulate IgG glycosylation.

MHC class II glycosylation modulates antigen presentation

The central event for initiation of T cell responses is $\alpha\beta$ T cell receptor ($\alpha\beta$ TCR) recognition of an antigenic epitope presented in the context of a class I or class II MHC (MHCI/MHCII) molecule. Most textbooks and articles diagram this interaction as that of a heterodimeric $\alpha\beta$ TCR protein associating with a heterodimer MHC molecule with a bound peptide antigen, which is based on numerous crystal structures of such ternary complexes (e.g., [44]). However, these structures are derived from recombinant molecules that lack their mammalian patterns of glycosylation. Although glycosylation has been thought of as inconsequential for both MHCI and MHCII, recent studies suggest that the glycans on both the $\alpha\beta$ TCR and MHCII can impact T cell activation.

$\alpha\beta$ TCRs are heavily glycosylated, with each subunit containing multiple acceptor sites for N-linked glycosylation. Glycan occupancy of one of these sites is needed for cell surface localization⁴⁵, suggesting a role in early ER quality control events. Due to the spatial orientation of $\alpha\beta$ TCR N-glycans, their potential role at the cell surface is thought to contribute to the orientation and organization of the molecule itself rather than to directly influence MHC interactions. For example, a global deficiency in the β 1,6 N-acetylglucosaminyltransferase V (Mgat5) enzyme (Figure 1) alters T cell N-glycosylation patterns resulting in a lower activation threshold *in vitro* and increased incidence of autoimmune disease *in vivo*⁴⁶. In this case, the absence of the Mgat5 enzyme, which is critical for normal T cell N-glycan branching (Figure 1), results in a lack of galectin-mediated $\alpha\beta$ TCR lattice formation (Box 2)⁴⁶. This is now known to be a critical feature of T cell receptor signaling, in part due to the role of CD45 within the $\alpha\beta$ TCR/galectin lattice (Box 2)⁴⁷. The cytoplasmic tail of CD45 contains phosphatase activity that maintains Lck, a critical signaling molecule that helps to initiate the cascade leading to T cell activation, in a de-phosphorylated and inactive state⁴⁸. The galectin-mediated lattice is responsible for holding CD45 and the TCR signaling complex in close proximity via their O- and N-linked glycans (respectively) to prevent low-avidity T cell activation^{13,46-48} (Figure 2).

Box 2

The Galectins and Cell Surface Lattices

Galectins are β -D-galactoside binding proteins (15 identified to date [58]) that are grouped into three classes based on global structure: prototypical, tandem-repeat, and chimeric (figure).⁵⁸ Cytoplasmic forms of galectins are typically found as monomers, whose function appears to rely upon protein-protein interactions more than protein-carbohydrate interactions that dominate at the cell surface. However, multimerized galectins seem to have a more potent signaling potential over their monomeric counterparts¹⁰². Several members of the galectin family seem to be ubiquitously expressed in cells of the immune system (e.g., galectin-1), whereas others are cell-type specific (e.g., galectin-10 is specifically expressed in eosinophils, basophils, and regulatory T cells) (see [58]).

The key mechanistic feature underlying galectin activity appears to be the ability to form multivalent protein-carbohydrate associations at the cell surface¹⁰³. As most membrane proteins have more than one O- or N-linked glycan per molecule, the multivalent nature of secreted galectins allows for a network of crosslinking events known as a lattice mediated by the surface glycoprotein glycans. A galectin lattice is analogous to lipid rafts

and other membrane structures that facilitate the formation of membrane domains that may be enriched for specific molecules while excluding others, only for galectins, this occurs by virtue of the fact that as secreted molecules, they contain at least two CRDs (figure). Within the immune system, clustering of surface receptors is a key to signal transduction leading to cellular activation, cellular inactivation, apoptosis, and other outcomes. As a result, galectins can both promote signaling through holding key molecules in close proximity or inhibit signaling by either excluding required molecules or through clustering inhibitory proteins with signaling molecules, as noted for $\alpha\beta$ TCR and CD45^{13,47}.

Galectin structural motifs

The galectin family is categorized by the structural arrangement of the carbohydrate recognition domains (CRDs). Prototypical galectins exist as monomers in the cytoplasm and are secreted as homodimers containing two CRDs (one per subunit). Tandem-repeat galectins are monomeric, yet contain two CRDs connected by a linker domain. Chimeric galectins exist as homopolymers with a variable number of subunits, each containing a single CRD.

Consistent with these findings, a recent study directly demonstrated that selective glycan modification of a tumor-specific $\alpha\beta$ TCR, through removal of glycan attachment sites, improved functional avidity of the T cell by improving $\alpha\beta$ TCR multimerization, decreasing TCR-MHC dissociation, and ultimately increasing the recognition of tumor cells bearing the target antigen⁴⁵. Importantly, site-directed mutagenesis of the TCR α -chain glycosylation sites alone influenced functional avidity of the T cell without conferring detectable self-antigen autoreactivity⁴⁵.

Both MHCI and MHCII molecules contain highly conserved acceptor sites of N-glycosylation across species, suggesting selective pressure that remains elusive. Historically, glycans on MHC have been thought to not play a significant role in peptide antigen binding

or in interactions with the $\alpha\beta$ TCR, and the only known role for the conserved N-glycan sites in MHCII is in protein folding and trafficking to the cell surface^{49,50}. However, data from studies of MHCII challenge this traditional viewpoint^{45,51}. The N-glycosylation of MHCII molecules does not appear to play a role in early protein folding events in the ER or in trafficking of MHCII through the secretory pathway. Instead, MHCII glycosylation can directly modulate antigen binding and presentation of an atypical class of T cell-dependent carbohydrate antigens (glycoantigens)⁵¹. Glycoantigens bind to MHCII at a relatively high affinity ($K_d = 315$ nM)⁵² in a size- and conformation-dependent fashion⁵³. Unlike peptide antigens, these glycoantigens fail to associate with MHCII molecules lacking mammalian complex N-glycans⁵¹. Expression of recombinant MHCII in cells with altered Golgi activity leads to glycan structural intermediates (*e.g.*, high mannose and hybrid-type glycans; Figure 1) on MHCII and a subsequent failure of MHCII to associate with glycoantigens *in vitro*. Likewise, ablation of the *Mgat2* locus (Figure 1) limits glycan branching and decreases glycoantigen presentation by MHCII leading to loss of T cell stimulatory activity⁵¹. Incomplete MHCII glycosylation does not appear to limit the capacity of MHCII to bind and present peptide antigens⁵¹, despite the fact that glycoantigen and peptide binding are competitive and probably share binding sites⁵². Given the relatively large size of MHCII-associated N-glycans (*i.e.*, roughly the size of an Ig domain each)⁵⁴, it is possible that the regulation of N-glycan structure, in particular the branching pattern (Box 1; Figure 1), could serve as a regulatory mechanism by which to manipulate antigen selection within the MHCII compartment. Although changes in N-glycan branching are associated with inflammatory disease (reviewed elsewhere [^{55–57}]) and are known to alter the presentation of at least one group of T-dependent antigens⁵¹, this intriguing possibility remains unexplored.

Galectins regulate leukocyte activity through the formation of lattices

Galectins are a highly conserved family of 15 known proteins initially identified based on their ability to bind β -D-galactosides, especially LacNAc (type I, Gal β 1-3GlcNAc; type II, Gal β 1-4GlcNAc)⁵⁸, found within both O- and N-glycans. These proteins function both intracellularly as modulators of the splicing machinery⁵⁹, and possibly of signaling cascades⁶⁰, as well as on the extracellular face of the cell, primarily through the formation of lattices with surface glycoproteins⁶¹ (Box 2).

The earliest work on this family of proteins dates back to 1975 where a β -D-galactoside binding lectin ('electrolectin') isolated from the electric eel *Electrophorus electricus* was discovered⁶², whereas the ability of galectins to modulate the immune response was first suggested in 1983 with the finding that electrolectin could prevent the onset of experimental autoimmune myasthenia gravis in rabbits.⁶³ Later, human galectin-1 (then known as IML-1) was shown to inhibit experimental autoimmune encephalomyelitis (EAE), a murine model of multiple sclerosis.⁶⁴ Since the renaming of this family to 'galectin' 1994¹², a number of key mechanistic studies have been published. First, in 1995⁶⁵ and in the subsequent three years^{66,67}, studies showed that galectin-1 is an important mediator of T cell apoptosis and is involved in homeostatic maintenance of T effector cell function. It was reported that galectin-3 is important in negatively regulating T cell activation through direct interactions with glycans decorating the $\alpha\beta$ TCR⁴⁶ (Figure 2).

Galectin binding has been linked to CD8⁺ T cell anergy after antigenic stimulation⁶⁸. Recent findings show that galectin-3: 1) destabilizes the immune synapse between professional antigen-presenting cells and responding T cells; 2) suppresses activation of early $\alpha\beta$ TCR signaling events; and 3) is involved in the endosomal sorting complex required for transport (ESCRT) of receptors needed for signaling and immune synapse formation.⁶⁹ T cell-mediated immunity can further be regulated through the interaction of

galectin-9 and Tim-3 on T helper type 1 (Th1) cells. This interaction is thought to regulate Th1 responses through inducing apoptosis of activated cells⁷⁰, and was also found to promote granulocytic myeloid-derived suppressor cell (MDSC)-mediated suppression of both CD4⁺ T cell proliferation and IFN γ secretion⁷¹. Regulatory IL-10-producing dendritic cells (DCs) were also induced upon exposure of immature DCs to galectin-1. These DCs promoted T cell tolerance and induced type 1 regulatory T (Tr1) cells in an IL-27-dependent fashion⁷² (Figure 2).

Using the EAE model, recent findings demonstrate that galectin-1 could be an important regulatory factor that limits autoimmune neurodegeneration. Administration of recombinant galectin-1 is known to ameliorate experimental autoimmune uveitis⁷³, a Th1-mediated inflammatory disease. Galectin-1 also deactivates classically activated, pro-inflammatory microglia through binding of CD45 at the cell surface⁷⁴ (Figure 2). This association appears to promote retention of CD45 at the plasma membrane, prolonging inhibitory phosphatase activity, deactivating the microglia and resulting in protection against immune-mediated neuronal damage and the onset of neurodegeneration.

The suppressive effects of galectins are not limited to tolerance or the regulation of Th1-mediated inflammation. Several other autoimmune conditions and cancers are associated with increased production of galectins and immune evasion. For example, the presence of galectins in solid tumors impairs the ability of anti-tumor T cells to effectively kill tumor cells. Although the mechanism is unclear, early studies indicate that treating mice with anti-galectin antibodies, galectin ligands, or metabolic inhibitors to eliminate host galectin binding show potential as therapeutics to overcome these suppressive effects^{75,76}. Beyond tumor immunology, results are less clear. Some viral infections, such as hepatitis C, induce elevated concentrations of galectins that induce Treg expansion and kill virus-infected cytotoxic T lymphocytes through crosslinking Tim-3⁷⁷. Other viral infections, such as herpes simplex virus (HSV), and some autoimmune conditions, potentially benefit from exogenous galectin therapy. In HSV-infected mice and in a mouse model of type 1-diabetes, galectin treatment promoted IL-10 secretion, triggered apoptosis of activated immune cells^{78,79}, impaired T cell homing, and decreased the secretion of pro-inflammatory cytokines⁷⁸. Similarly, oral GlcNAc administration enhances N-glycosylation, including galactosylation, thereby increasing the ligands available for surface galectins and inhibiting Th1/Th17 activity in EAE⁸⁰. Consistent with these data, recent reports show that a number of factors, such as host metabolism and vitamin D(3), can contribute to altered glycan synthesis and lead to autoimmunity^{81,82}, including EAE. In fact, mouse strains susceptible to EAE (i.e., PL/J, SJL, and NOD) show differences in N-glycan branching patterns⁸³ (see Figure 1), which is known to influence galectin binding affinity⁸⁴ and therefore lead to changes in galectin-mediated lattice formation and T cell-dependent neurodegeneration; however, the relevance to human multiple sclerosis remains unclear.

Although some galectins can act as suppressive molecules in inflammation and T cell responses, not all members of the galectin family follow the same pattern. For example, in contrast to the observation that galectin-3 prevents low-stringency activation of the $\alpha\beta$ TCR, galectin-8 binding to T cells appears to promote T cell proliferation, possibly through unique interactions with CD45⁸⁵ (Figure 2). The varied effects of galectins indicate differences in their binding specificities and downstream signaling pathways. It remains unclear how these glycan binding proteins have specific effects at the cell surface despite the fact that their minimal carbohydrate ligand is present on many or even most of the surface proteins on that same cell. Although binding studies are beginning to identify particular glycan ligands beyond LacNAc which are recognized by specific galectins⁸⁶, more work is needed to clarify the mechanisms by which galectins associate with their glycan targets and produce specific immune effects.

C-type lectins are pro- and anti-inflammatory immunomodulators

C-type lectin receptors (CLRs) are another group of glycan-binding proteins that are important for immune recognition and regulation. CLRs were originally characterized as a family of Ca^{2+} -dependent cell surface proteins, although this family now includes homologous but calcium-independent receptors. As a key member of the pattern recognition receptor (PRR) superfamily, CLRs bind a variety of glycan structures including mannose, fucose, galactose, and β -glucan polymers on fungi. These lectins play important roles as adhesion molecules, signaling molecules, and antigen uptake mediators (reviewed in [58,87]). Polymorphisms in several CLR genes associate with autoimmune diseases, such as type 1 diabetes and multiple sclerosis^{88,89}.

Some CLRs, such as the secreted RegIII γ , have direct bactericidal properties. Systemic administration of flagellin stimulates TLR5 on CD103⁺CD11b⁺ lamina propria DCs, resulting in IL-23 production which stimulates IL-22 and RegIII γ production by the intestinal epithelium⁹⁰ (Figure 2). By associating with glycans, RegIII γ binds to peptidoglycan of gram-positive bacteria leading to microbial cell death⁹¹. Although this lectin pathway increases defense against some bacterial pathogens, *Helicobacter pylori* was recently shown to induce RegIII γ via production of the CagA cytotoxin, which stimulates the gastric epithelium in a STAT3-dependent fashion⁹². This could be a mechanism by which gram-negative *H. pylori* reduces its microbial competition through the induction of gram-positive bactericidal activity⁹².

In contrast to RegIII γ , most CLRs are cell surface transmembrane proteins. Dectin-1 (also called CLEC7A) is an important CLR that recognizes β -glucans (i.e., β -linked polymers of glucose) on the surface of fungi and triggers key antimicrobial pathways, such as the respiratory burst and phagocytosis⁹³. Only insoluble β -glucans activate Dectin-1-dependent signaling, consistent with the recognition of fungi-attached β -glucans rather than soluble fragments⁹³. Dectin-1-deficient mice show greater sensitivity to dextran sulfate sodium (DSS)-induced colitis. This is linked to loss of direct contact between dectin-1 and the β -glucans found on fungi of the normal commensal flora, because DSS-induced colitis was reduced in dectin-1-deficient animals after treatment with anti-fungal antibiotics (i.e., fluconazole).⁹⁴ A polymorphism in the human CLEC7A (dectin-1) gene was linked to a severe form of ulcerative colitis, suggesting that the mouse observations are directly relevant to human inflammatory bowel disease.⁹⁴

CLRs represent potential targets for vaccine design. CLEC9A is a recently discovered member of the CLR family that is expressed on a small proportion of DCs, B cells, and monocytes. Human myeloid DCs expressing this receptor are able to cross-present antigens delivered via anti-CLEC9A antibodies, leading to both CD4⁺ and CD8⁺ antigen-specific T cell responses⁹⁵. Other members of this family that have been well studied are DC-SIGN and DC-ASGPR, both of which are found primarily on DCs. These molecules have become popular targets for therapeutics because of their role in the generation of antigen-specific T cell responses. Targeting antigens directly to DC-ASGPR promotes the activation and differentiation of regulatory T cells that secrete IL-10⁹⁶, while oral treatment of mice with highly mannosylated molecules target murine DCs through SIGN-R1, the mouse homolog to DC-SIGN, and leads to oral tolerance and the induction of IL-10 secretion⁹⁷. Remarkably, the signaling outcome downstream of DC-SIGN appears to be sensitive to the glycan composition of the ligand, which contradicts the common assumption that signaling through most cell surface receptors is independent of the ligand and can be studied using non-physiologic ligands (e.g., antibody-mediated crosslinking). A recent study examined the engagement of DC-SIGN by *Mycobacterium tuberculosis*, HIV-1, and *H. pylori* as well as the cytokines induced by this activation⁹⁸. Although all ligands bound to DC-SIGN,

mannose-rich agonists expressed by *M. tuberculosis* and HIV-1 led to the expression of pro-inflammatory molecules such as IL-12 and IL-6, whereas fucose ligands from *H. pylori* primarily elicited only IL-10⁹⁸ (Figure 2). These data demonstrate that DC-SIGN-mediated signaling can be tuned to a pro- or anti-inflammatory status based on the glycan composition. However, DC-SIGN is not only important for the recognition of glycan ligands from microbial sources, but it also appears to play a role for the function of IgG antibodies as discussed earlier⁹⁹.

Concluding remarks

The advances in glycoimmunology highlighted here illustrate the inescapable conclusion that glycosylation plays a regulatory and often pivotal role in the immune system. In broad terms, pathways that influence glycan structure and activity, as well as pathways that influence glycan-binding proteins can both influence immunity. Changes in glycan structure are linked to inflammation and infection, as illustrated by regulation of IgG function by galactosylation^{35,37} and sialylation^{26,33,34}, and by regulation of antigen presentation by MHCII glycosylation⁵¹. However, the nature of the relationship between inflammation or infection and glycan structure, the impact on immune function, and whether glycan changes are a consequence or an underlying cause of the inflammatory cascade remain unclear⁵⁵.

While the regulation of glycan structure also directly impacts glycan-binding protein activity, the biological function of these molecules appears to be context-dependent. For example, DC-SIGN signaling is influenced by simultaneous Toll-like receptor (TLR) ligation on the same cell¹⁰⁰, which is consistent with the idea that a combination of PRR signaling events on the same cell converge to determine outcome. Galectin function also seems context dependent and dictated by the glycoprotein to which the galectin is bound⁵⁸, rather than by the galectin itself. A thorough understanding of how glycan-binding proteins achieve specific and differential responses under a variety of normal and disease-associated states remains a central issue.

There are at least two main challenges in the field. First, regulation of protein glycosylation is poorly understood, in particular regulation of glycosylation on a specific protein rather than the glycome of an entire cell. Golgi enzyme activity, sugar-nucleotide transporter expression, metabolism of precursor carbohydrates, and trafficking of nascent proteins through the ER and Golgi could all regulate glycosylation, but the pathways responsible for such regulation and the immunological signals they respond to are enigmatic. A second challenge is that experimentally manipulating glycan structures on a specific protein in a living cell or organism is impossible, short of simply removing the glycosylation site, which could have very different effects compared to alterations in the glycan structures themselves. A 'systems' approach is probably necessary to truly integrate changes in cellular glycosylation with cellular function rather than focusing on single molecules.

Approaching these questions is becoming easier due to significant technological progress dissecting glycan compositions and structures. Core facilities at the University of California San Diego (<http://glycotech.ucsd.edu/>), the University of Georgia Complex Carbohydrate Research Center (<http://www.ccrcc.uga.edu/services/index.php>) and elsewhere are now available to help with glycan-related analyses on a fee-for-service basis. The Consortium for Functional Glycomics (CFG; <http://www.functionalglycomics.org/>) has been responsible for developing glycan binding arrays to determine glycan binding specificity, while making a large variety of purified and synthesized glycans available for investigators. The glycosylation patterns found on many common cell lines, primary cells, and tissues are freely available in the CFG-maintained databases. These technical advances coupled with

increased availability of services and reagents reduce the technical threshold for entry into the intersection of glycobiology and immunity.

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Figure 1. Protein Glycosylation and Important Glycan Epitopes

Schematic representations of N-linked, O-linked (O-GalNAc), and O-GlcNAc protein glycosylation pathways. For the N-linked glycosylation pathway, the structures falling into ER-localized, high mannose, hybrid, and complex groups are indicated to illustrate the general steps of synthesis and structural diversification that occurs in the Golgi apparatus. Differences in branching patterns are also illustrated, together with details about the enzymes (Mgat1–5) responsible for the addition of GlcNAc residues that seed each branch. Branching is a modulated structural attribute of N-glycans during disease and inflammation, which can be regulated through differential expression of the Mgat genes. For the O-linked glycosylation pathway, each of the “Core” structures are shown to illustrate the structural relationships between each glycan type. All of the “Core” structures can serve as a platform for further diversification, as shown for Core 1 and Core 3. Within both N- and O-linked glycans, a number of key glycan epitopes and ligands that are relevant for the immune system are possible and indicated in red boxes. For example, sialyl-Lewis^x is a ligand for selectin molecules, whereas LacNAc and poly-LacNAc represent minimal glycans for many of the galectins. We also show both Type-1 and Type-2 A/B/O(H) blood group antigens which can also be found in both N- and O-linked glycans as examples relevant to transplantation and blood transfusion.

Figure 2. Pathways for immune regulation by glycans and glycan-binding proteins

Glycan binding proteins, such as the galectin and C-type lectin families, play both positive and negative roles in the induction of an immune response. For example, galectin-1 (Gal-1) and galectin-3 (Gal-3) hold CD45 at the cell surface, such that T cell receptor signaling is muted by preventing Lck phosphorylation, while Gal-1 can also impact the differentiation of the FoxP3⁺ Tr1 subset of inducible T regulatory cells through an unknown mechanism. The C-type lectin DC-SIGN functions not only to recognize foreign carbohydrates from *H. pylori* and *M. tuberculosis*, but it also binds to sialylated IgG molecules (sIgG) as part of an anti-inflammatory cascade. Galactosylated IgG (gIgG) crosslinks the inhibitory FcγRIIB receptor with Dectin-1, which leads to inhibition of C5aR signaling, whereas galectin-9 associates with glycans on Tim-3 leading to apoptosis. The glycans themselves can regulate these interactions through programmed changes in glycan structure and diversification, which could be mediated by changes in expression of glycosylation-specific Golgi enzymes or their carbohydrate substrate concentrations.