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Glycomaterials for immunomodulation, immunotherapy, and infection prophylaxis

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Synthetic materials that can engage the innate and adaptive immune systems are receiving increasing interest to confer protection against onset of future disease, such as pathogen infection, as well as to treat established diseases, such as autoimmunity and cancer. Carbohydrates are integral to various immune-related processes, including inflammation, adaptive memory, and tolerance, through both their non-covalent recognition of carbohydrate-binding proteins and as chemical signatures that distinguish self from non-self. Harnessing the biological activity of carbohydrates, however, was long hindered by the lack of a 'sugar code' defining their structure-function relationships, the difficulty of carbohydrate synthesis, and the weak binding affinity of monovalent carbohydrates for proteins or other biomolecules. The advent of new glycan synthesis approaches, combined with increased understanding of the role of multivalent carbohydrate clusters in immunology, has spurred significant recent growth in the development of multivalent synthetic materials modified with carbohydrates (i.e. "glycomaterials") to activate, temper, or inhibit specific immunological processes. In this review, we highlight recent advances in glycomaterials that can inhibit T cell apoptosis, establish antigen-specific tolerance, suppress inflammation, or inhibit viral entry into host cells via non-covalent recognition of carbohydrate-binding proteins. In addition, we survey glycomaterials that can act as vaccines for adaptive immunological recognition and memory of carbohydrate antigens, with a particular emphasis on vaccines against tumor-associated carbohydrate antigens as cancer immunotherapies. In total, these examples demonstrate the enormous potential of glycomaterials to prevent or treat diverse diseases by engaging specific immunological processes.

1. Introduction

Carbohydrates, including monosaccharides, oligosaccharides, polysaccharides, and their conjugates, are major components of all organisms. For example, cellulose, a polymer of β-linked D-glucose found in plants, algae, and certain bacteria, is the most abundant organic compound on Earth.[1] In addition, approximately 50% of mammalian proteins are post-translationally modified with mono- or oligosaccharides (i.e. "glycosylated").[2] Although long-thought to serve primarily passive space-filling or barrier roles, there is growing appreciation that carbohydrates are integral to numerous biological processes in accordance with their natural abundance. For example, non-covalent interactions between carbohydrates and carbohydrate-binding proteins are essential for gamete fusion during the earliest stages of development in various organisms,[3] as well as maternal tolerance of the fetus throughout the course of mammalian development.[4] The importance of carbohydrates in human development is further exemplified by "congenital disorders of glycosylation", rare defects in carbohydrate synthesis machinery that are associated with a range of minor to severe developmental and functional consequences.[5] In addition to their role in development, carbohydrates and their interactions with carbohydrate-binding proteins are also integral to inflammation,[6] immune privilege in various adult tissues (e.g. cornea and central nervous system),[7,8] pathogen infection ,[9] wound healing,[10] and metastasis.[11] Thus, carbohydrates are intriguing as both targets and bioactive components of therapeutics and diagnostics for various biomedical applications. Here, we focus on recent advances in synthetic materials modified with carbohydrates, referred to as "glycomaterials", as therapeutics that can engage the immune system to prevent or treat diseases.

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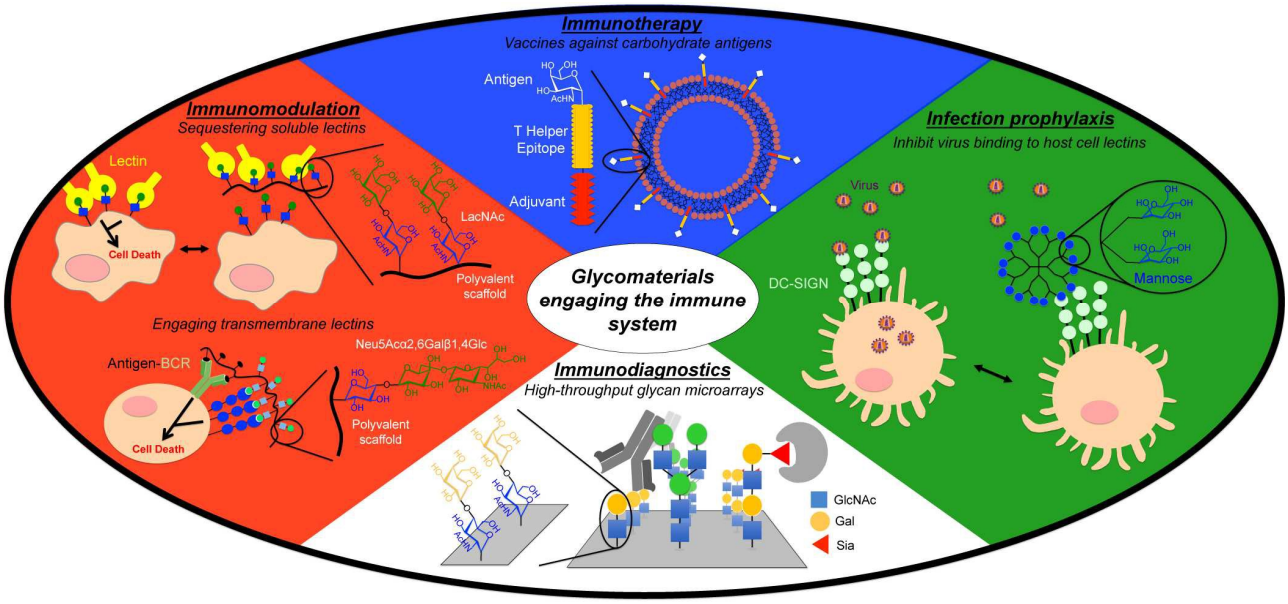

Figure 1: Examples of glycomaterials for immunotherapy, immunomodulation, infection prophylaxis, and immunodiagnostics highlighted in this review.

Synthetic materials are receiving increasing interest for creating carbohydrate-based therapeutics because they provide platforms to mimic immobilized multivalent carbohydrate clusters that are prevalent in nature. For example, mammalian epithelial cell surfaces are rich in glycosylated proteins (i.e. "glycoproteins"), glycolipids, and polysaccharides, which form the "glycocalyx", a viscous, gel-like sugar coating.[12] In addition, mammalian extracellular matrices are composed of glycoproteins, polysaccharides, and proteoglycans organized into a hierarchically-ordered network with tissue-specific compositional, structural, and functional properties.[13,14] Similarly, microbial cell surfaces have a glycocalyx that is primarily composed of polysaccharides, such as the capsular polysaccharides (CPS) and lipopolysaccharides (LPS) of the bacterial cell wall,[15] while bacterial "biofilms" are supported by an extracellular matrix of polysaccharides associated with DNA and proteins.[16] Finally, glycoproteins are often embedded within the phospholipid membrane surrounding enveloped virus protein capsids.[17] Often referred to as the 'glycocluster effect',[18] the immobilization, valency, and spatial organization of glycans within natural microenvironments are important determinants of carbohydrate biological activity. In particular, glycan immobilization and valency can significantly increase the otherwise low, micro- to millimolar range affinity of monovalent carbohydrates for proteins and other biomolecules through avidity effects, such as chelation and statistical rebinding.[19] In addition, valency and spatial organization can introduce physical or steric constraints that influence carbohydrate binding to biomolecules.[20,21] In this review, particular emphasis is placed on the ability of synthetic glycomaterials to recapitulate natural glycocluster effects via multivalency, while also providing exceptional control of glycan type, density, and organization to optimally activate, temper, or inhibit specific immunological processes. In particular, the following sections will survey synthetic multivalent glycomaterials that can modulate innate and adaptive immune responses via binding to specific carbohydrate-binding proteins, act as vaccines for cancer immunotherapy, confer protection against viral entry into host immune cells, and screen for carbohydrate-binding biomolecule expression (Figure 1).

2. Glycomaterials for immunomodulation

Non-covalent interactions between glycans and "lectins", a diverse family of soluble and transmembrane carbohydrate-binding proteins, can modulate or reshape various aspects of innate and adaptive immunity. For example, binding of soluble galectins to β-galactosides on cell surface and extracellular matrix glycoproteins can activate *outside-in* signaling events that influence various immunological processes, including inflammation,[22] auto-reactive T cell 'negative selection' in the thymus,[23] and antigen-specific T cell activation.[24] Binding of transmembrane Sialic acid-binding immunoglobulin-type lectins, or "Siglecs", to sialic acids that are prevalent on mammalian glycoproteins and glycolipids is important for B cell recognition of 'self' and natural killer cell recognition of 'damaged self'.[25] In contrast, binding of C-type lectin receptors (CLR) to foreign carbohydrates, such as mannose, provides an innate immune system mechanism for detection of 'non-self' that can amplify foreign antigen internalization, processing, and presentation to T cells.[26] Finally, binding of E-selectin to sialyl Lewis X (SLeX) mediates leukocyte adhesion, rolling, and extravasation through endothelium into damaged tissue sites during inflammation.[27] As a result, there is growing interest in therapeutics that can disrupt lectin-glycan interactions to promote or suppress particular immunological events.

Perturbing lectin-glycan interactions is often hindered by the low affinity of monovalent carbohydrates for highly-

hydrated lectin carbohydrate recognition domains (CRDs). This can be addressed, in part, with polysaccharides or synthetic glycoclusters that recapitulate the avidity effects of high-density glycans found throughout nature.[28] However, additional challenges arise because different lectins within a particular family can have antagonistic or specialized roles in innate or adaptive immunity, yet their CRDs are often highly conserved and therefore difficult to selectively target. For example, galectin-1, -3, and -8 preferentially bind β-galactosides, yet galectin-1 and galectin-8 can co-stimulate antigen-specific T cell responses, whereas galectin-3 antagonizes galectin-1 and galectin-8 signals.[29] Siglec-2 and Siglec-7 demonstrate preference for α2,6-linked sialic acids, yet Siglec-2 is exclusively expressed by B cells and regulates their activation during B cell receptor (BCR) recognition of antigens, whereas Siglec-7 regulates natural killer cell-induced cytotoxicity.[30,31] Observations from natural lectin-glycan interactions suggest that binding affinity and specificity are influenced by carbohydrate multivalency, as well as the spatial organization of carbohydrates within glycoclusters. For example, dense clusters of complex-type (CX) β-galactosides on the HIV gp120 coat protein can bind to galectin-1, which stabilizes gp120 interactions with host CD4 molecules.[32] In contrast, galectin-3 is unable to bind to gp120 CX glycans in their native conformation.[32] Thus, there is growing interest in synthetic glycoclusters that provide exquisite control of carbohydrate type, density, and spatial organization to selectively disrupt lectin-carbohydrate interactions. For example, a recent survey of synthetic multivalent galectin inhibitors, such as glycodendrimers, calix[n]arenes, cyclic glycopeptides, and cyclophanes, underscores the importance of glycan valency and spatial organization in galectin inhibitor design, particularly in the context of galectin binding to cancer cell surface glycans.[33] Here, we focus on synthetic glycomaterials that provide control of glycan type, valency, and spatial organization to modulate the bioactivity of galectins, Siglecs, CLRs, and selectins for immunomodulation.

2.1 Glycomaterials that sequester galectins to inhibit T cell apoptosis

Galectins are often over-expressed within tumor microenvironments and at the fetal-maternal interface, where they can induce activated T cell apoptosis and establish local sites of immunological privilege.[34,35] Thus, there is increasing interest in therapeutics that can perturb galectin-T cell binding within disease microenvironments to maintain local T cell-dependent immune responses. Small molecules, such as β-galactoside analogs and lactosamines,[36,37] are often hindered by low galectin-binding affinity and selectivity. Naturally-derived polysaccharides, such as modified citrus pectin (MCP) and Davanat® (Galectin Therapeutics),[38,39] often demonstrate higher galectin-binding affinities than small molecules, but afford limited control of ligand density and organization. In the following sections, we highlight recent examples of self-assembled glycomaterials with easily adaptable carbohydrate composition that can be tailored to enhance galectin-binding affinity and specificity, emphasizing the efficacy of these glycomaterials as inhibitors of T cell response to galectins.

2.1.1 Pseudopolyrotaxanes. To create glycomaterials with optimal galectin-binding properties, Stoddart, Baum, and colleagues adapted a pseudopolyrotaxane supramolecular assembly originally developed by Harada.[40,41] In particular, they synthesized lactose-modified cyclodextrin (LCD) "beads" that are threaded onto polyviologen polymer "strings". Self-assembled LCD "beads on a string" inhibited galectin-1-mediated T cell agglutination, an early marker of apoptosis, more effectively than soluble lactose or LCD units (10- and 6.7-fold, respectively). In contrast, a chitosan-based glycomaterial only provided 1.7-fold enhancement over soluble lactose, while trivalent glycoclusters showed no enhancement of inhibitory efficacy over soluble lactose. The authors suggested that the enhanced inhibition of galectin-1 by supramolecular assemblies was due to the ability of the LCD to freely rotate around the polyviologen axis, as well as slide along its backbone, which enabled dynamic rearrangement of the glycan ligands into the most thermodynamically-favorable spatial organization for galectin binding.

In a subsequent study, varying the length of the polyviologen backbone and number of LCD units threaded onto the polymer provided insights into correlations between the physical characteristics of pseudopolyrotaxane LCD self-assemblies and their interaction with galectin-1.[42] For example, supramolecular assemblies were more effective inhibitors of T cell agglutination via galectin-1 than soluble LCD or soluble β-lactose, regardless of polymer length or number of LCD units, underscoring the importance of multivalency and cooperative ligand binding for galectin inhibition. Assemblies with a longer polyviologen backbone inhibited T cell agglutination more effectively than those consisting of a shorter polymer, suggesting that galectin-glycan binding is enhanced by a greater number of total ligands in a given supramolecular assembly and/or a greater distance for intramolecular bridging of the polymer via galectin-1 dimers. Interestingly, assemblies with roughly 25% of polyviologen sites "threaded" by LCD inhibited T cell agglutination by galectin-1 more effectively than half-threaded and nearly completely threaded assemblies, demonstrating that maximal ligand valency does not necessarily result in highest binding affinity. Taken together, these observations demonstrate the inherent advantages of highly multivalent glycomaterials with tunable valency characteristics for creating galectin inhibitors with optimal properties.

2.1.2 Self-assembled glycopeptide nanofibers. Our laboratory recently created a multivalent galectin inhibitor based on self-assembled glycopeptide nanofibers.[43] In particular, we synthesized a glycosylated analog of the β-sheet fibrillizing peptide Q11 (QQKFQFQFEQQ), which self-assembles into nanofibers under aqueous conditions (Figure 2a).[44] The concentration of carbohydrate integrated into the nanofibers can be easily and precisely varied by simply mixing different molar ratios of glycosylated and non-glycosylated Q11 molecules together in the pre-assembled state. In addition,

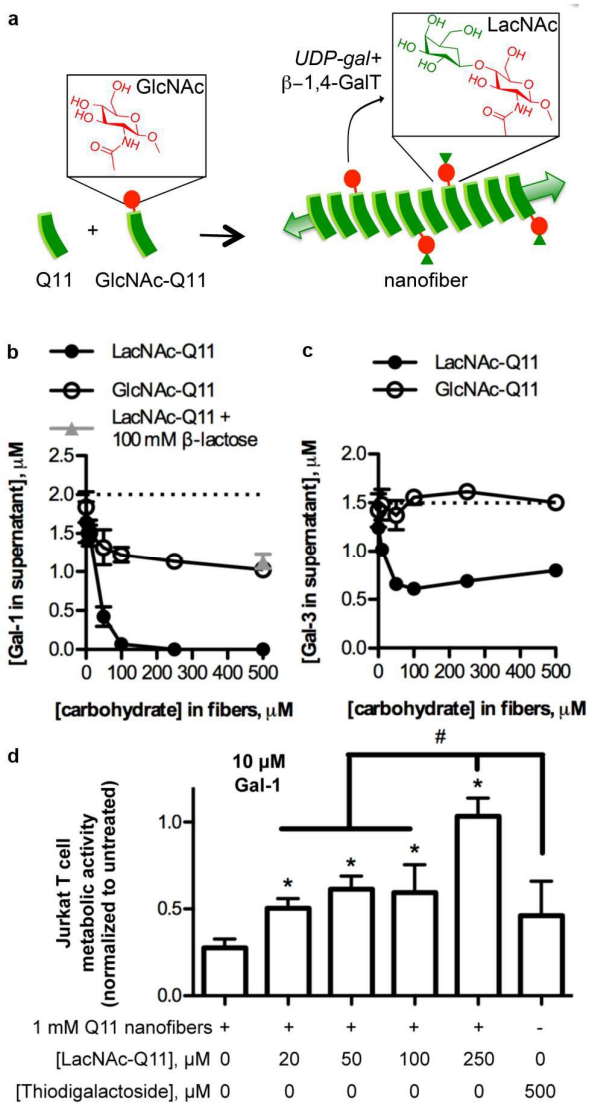

Figure 2: Self-assembled glycopeptide nanofibers that sequester galectin-1 to inhibit T cell apoptosis. A) Schematic representation of glycopeptide self-assembly into nanofibers with tailored carbohydrate content and chemistry. B) Binding of galectin-1 and (C) binding of galectin-3 to GlcNAc-Q11 and LacNAc-Q11 nanofibers. D) Inhibition of galectin-mediated T cell apoptosis by LacNAc-Q11 nanofibers. Reproduced with permission from [43].

glycan chemistry can be tailored by incubating glycopeptide nanofibers with a glycosyltransferase and sugar donor, as exemplified by the efficient *on-nanofiber* conversion of GlcNAc monosaccharides to galectin-binding LacNAc disaccharides.

Self-assembled glycopeptide nanofibers bound galectins in a carbohydrate-dependent manner. In particular, affinity of LacNAc-Q11 nanofibers for galectin-1 increased with LacNAc-Q11 mole fraction over the range of 0-50%, whereas galectin-3 binding was saturated at LacNAc-Q11 mole fractions $\geq$ 10% (Figure 2b-c). Notably, glycan chemistry was an important determinant of galectin-binding affinity, since GlcNAc-Q11 nanofibers had lower affinity for galectin-1 than LacNAc-Q11 nanofibers, and no apparent affinity for galectin-3 (Figure 2b-c). In contrast, affinity of GlcNAc-Q11 nanofibers for wheat germ agglutinin (WGA), a GlcNAc-binding lectin, correlated with GlcNAc-Q11 mole fraction, whereas LacNAc-Q11 nanofibers demonstrated no apparent affinity for WGA. Thus, the ability to easily and precisely tailor the type and valency of carbohydrate ligands displayed by self-assembled glycopeptide nanofibers enabled fine tuning of their lectin-binding properties.

LacNAc-Q11 nanofibers inhibited T cell apoptosis via galectin-1 as measured by agglutination, phosphatidylserine exposure, and metabolic activity loss. As expected, nanofibers with the highest galectin-1 binding affinity (i.e. highest LacNAc-Q11 mole fraction) inhibited T cell apoptosis most effectively, and were significantly more potent than the monovalent β-galactoside analog, thiodigalactoside (TDG) (Figure 2d). This enhanced inhibition of T cell apoptosis was likely due to the nearly order of magnitude increase in binding affinity of galectin-1 for nanofibrillar LacNAc when compared to soluble lactose. Notably, LacNAc-Q11 nanofibers failed to inhibit galectin-3 mediated T cell apoptosis, consistent with data demonstrating that LacNAc-Q11 nanofibers have higher affinity for galectin-1 than galectin-3. Taken together, these observations suggest that self-assembled glycopeptide nanofibers with easily modifiable carbohydrate content may ultimately lead to galectin inhibitors having binding affinity and selectivity that greatly surpasses that of conventional small molecule or polysaccharide-based inhibitors.

2.2 Glycomaterials that engage Siglecs to induce B cell tolerance

The primary function of B cells is to produce antigen-specific antibodies in response to antigen detection via B cell receptors (BCRs), a diverse population of membrane-anchored antibodies with highly variable antigen-binding domains.[45,46] BCR recognition of foreign antigens is therefore crucial for establishing humoral immunity to pathogens, yet aberrant BCR recognition of self-antigens can lead to autoimmunity. Siglec-2 and Siglec-G are key regulators of B cell autoantibody production, which co-localize with BCR via binding to "self" sialic acids in *cis* (i.e. on the same cell) or *trans* (i.e. on a neighboring cell), and in turn inhibit intracellular signal transduction following BCR recognition of autoantigens.[47] As a result, there is growing interest in creating therapeutics that can engage Siglec-2 and Siglec-G to modulate B cell activation.

Following the general trend of inhibitors of lectin-carbohydrate interactions, creating therapeutics to modulate Siglec function is also challenged by the low affinity of Siglecs for monovalent sialic acids (e.g. Neu5Acα(2,6)Galβ, Kd ≈ 0.2 mM).[48] In addition, therapeutics to activate Siglecs in *trans* must also overcome natural *cis* interactions between Siglecs and dense glycans present on the host cell membrane. For example, Razi and Varki demonstrated that glycopolymers modified with natural sialic acid ligands could only engage Siglec-2-positive B lymphoma cells or resting human peripheral blood B cells in *trans* if *cis*-acting sialic acids were first removed

from the cell surface.[49] Since then, sialic acid glycomaterials based on polymers, liposomes, and virus-like particles (VLPs) have demonstrated efficacy for engaging Siglec-2 in *trans* to deliver payloads that induce cell apoptosis as a treatment for B cell lymphoma.[50,51,52] Below, we highlight advances in synthetic glycomaterials engineered to engage Siglecs in *trans* to induce antigen tolerance.

2.2.1 Sialylated polymers to induce antigen-specific B cell tolerance. To understand the role of Siglec-2 *trans* interactions on antigen-specific B cell activation in vitro, Kiessling and colleagues created co-polymers of a Siglec-2 ligand, Neu5Acα2,6Galβ1,4Glc, and the T-independent hapten, dinitrophenol.[53] Polymers having only the Siglec-2 ligand did not bind B cells, whereas those having only the hapten potently activated B cells, as indicated by increased intracellular calcium flux and phosphorylation of tyrosine kinases associated with BCR signaling. In contrast, co-polymers of the hapten and Siglec-2 ligand bound to B cells and attenuated BCR signaling, thereby inhibiting B cell activation. This study provided an important early demonstration that polyvalent sialic acid glycomaterials can engage Siglec-2 in *trans* to inhibit antigen-specific B cell activation.

Following from this initial study, Paulson, Nemazee, and colleagues demonstrated that polymer conjugates carrying natural or synthetic glycan ligands that bind both Siglec-2 and Siglec-G can induce antigen-specific B cell tolerance to a T-independent hapten (nitrophenol (NP)) in vivo.[54] In particular, polymers carrying only the hapten induced robust anti-NP immunoglobulin (Ig)M and IgG3 antibodies, whereas polymers carrying the hapten and a natural or synthetic Siglec ligand induced weak or undetectable anti-NP responses, respectively. Notably, a single dose of hapten-polymer conjugate carrying the synthetic Siglec ligand induced persistent NP tolerance, whereas the molar ratio of hapten to natural Siglec ligand governed tolerance induction. In the presence of a Toll-like receptor (TLR)-4-binding adjuvant, a hapten-polymer conjugate carrying the synthetic Siglec ligand significantly reduced anti-NP responses, despite failing to induce tolerance, demonstrating that engaging Siglecs in *trans* can suppress B cell activation in response to inflammatory stimuli.

2.2.2 Siglec-engaging tolerance-inducing antigenic liposomes. Paulson and colleagues created liposomal vehicles capable of inducing tolerance to both T-independent haptens and T-dependent protein antigens by engaging Siglecs in *trans*.[55,56] For example, Siglec-engaging tolerance-inducing antigenic liposomes, or "STALs", modified with NP or hen egg lysozyme induced tolerance to either antigen by up-regulating antigen-specific B cell apoptosis.[55,56] In addition, STALs engaging Siglec-2 also induced tolerance to ovalbumin, myelin oligodendrocyte glycoprotein, and Factor VIII (FVIII).[55] FVIII-STAL enhanced the efficacy of recombinant FVIII for inhibiting blood loss in a murine model of hemophilia,[55] which is noteworthy since long-term clinical use of recombinant FVIII is often limited by production of inhibitory antibodies. Thus, STALs may be able to establish prophylactic tolerance that can enhance the clinical efficacy of inherently immunogenic biotherapeutics.

2.3 Targeted antigen delivery via CLRs

Ligands that bind to receptors that are exclusively expressed by antigen-presenting cells (APCs) can provide targeted antigen delivery that greatly amplifies antigen immunogenicity.[57,58] Carbohydrates are particularly interesting as targeting ligands, because dendritic cells (DCs) and macrophages exclusively express numerous transmembrane CLRs that bind to carbohydrates and internalize them via receptor-mediated endocytosis.[59] As a result, antigens targeted to CLRs via carbohydrate ligands can more efficiently enter the endo-lysosomal pathway, where they are processed and loaded onto major histocompatibility complex (MHC) class II molecules for presentation to CD4+ T cells.[60] In addition, targeting antigens to CLRs can also induce cross-presentation to generate CD8+ cytotoxic T cell responses and long-term T cell memory through a yet undefined pathway.[61]

APCs express numerous different CLRs, including DEC205, DC-SIGN, Dectin-1, mannose receptor (MR), langerin, and macrophage galactose-type lectin (MGL).[59] Initial efforts to target CLRs focused on receptor-specific antibodies.[60] However, each CLR contains a unique carbohydrate-recognition domain with specificity for different carbohydrate structures, such as mannose, fucose, or galactose. Thus, as understanding of the carbohydrate-binding specificity of each receptor has increased, so have efforts to develop glycomaterials that can selectively target antigens to specific CLRs. In the following sections, we highlight recent developments in materials-based approaches for targeted antigen delivery to DC-SIGN, mannose receptor, and Dectin-1.

2.3.1 DC-SIGN targeting. DC-SIGN is one of the best understood CLRs.[62] It contains a carbohydrate-recognition domain that has specificity for high-mannose-containing structures and Lewis-type blood antigens (i.e., Le^X, Le^Y, Le^b and Le^a). Early studies demonstrated that modifying soluble antigens with DC-SIGN-binding glycans can enhance their uptake, processing, and presentation to CD4+ T cells.[63] Based on these observations, van Kooyk and colleagues developed materials-based approaches to enhance antigen immunogenicity via DC-SIGN targeting. In one approach, they created poly(amido amine) (PAMAM) dendrimers modified with a peptide antigen terminated with Le^b.[62] Dendrimer binding to DC-SIGN increased with Le^b multivalency, with dendrimers carrying 16-32 glycan units demonstrating optimal internalization by DCs, routing to lysosomal compartments, antigen presentation, and induction of antigen-specific CD4+ and CD8+ T cell proliferation.

In an alternative approach, van Kooyk and colleagues developed liposomes modified with the DC-SIGN-binding glycans, Le^b or Le^X.[64] DC-SIGN targeting increased DC internalization of liposomes, DC presentation of encapsulated antigen to CD4+ and CD8+ T cells, and differentiation of transgenic antigen-specific CD8+ T cells into interferon (IFN)-γ-producing effector cells in vitro. Notably, targeting was specific

for DC-SIGN, as anti-DC-SIGN antibodies reduced antigen presentation. Le^x-modified liposomes also enhanced CD14+ dermal DC presentation of melanoma antigens to antigen-specific CD8+ T cells by enhancing liposome internalization via DC-SIGN.[65] However, liposome internalization and antigen presentation by dermal DCs required co-administration with cytokines, GM-CSF and IL-4, to upregulate dermal DC expression of DC-SIGN. More recently, a TLR-4-binding adjuvant (e.g. monophosphoryl lipid A (MPLA)) integrated into Le^x-modified liposomes provided "self-adjuvanting" vaccines that induced DC activation and pro-inflammatory cytokine expression in a DC-SIGN-dependent manner, and in turn, significantly enhanced DC cross-presentation of a melanoma antigen to antigen-specific CD8+ T cells.[66]

Liposome formulation is an important determinant of CLR targeting. For example, DCs internalized glycoliposomes with a poly(ethylene glycol) (PEG) spacer separating the particle and glycan to a lesser extent than glycoliposomes lacking PEG.[67] Multivalent glycosylated liposomes were efficiently internalized via DC-SIGN but not Langerin, which instead preferentially internalized monovalent glycopeptides.[65] It has been suggested that these observed differences in CLR-mediated internalization are due to differences in receptor clustering into nanodomains within the cell membrane, atlhough many mechanistic details remain to be understood. Nonetheless, glycan presentation and the 'glycocluster effect', in which multivalency can increase protein-carbohydrate binding affinity while also introducing steric limitations that confer protein-carbohydrate binding specificity, are likely to be important design considerations when creating new glycomaterials to target CLRs.

In addition to their potential to enhance induction of therapeutic or prophylactic immunity, glycomaterials targeting DC-SIGN can also aid in elucidating the molecular mechanisms of CLR-mediated antigen internalization, processing, and presentation. For example, Cambi et al. created quantum dots (QDs) modified with SLeX glycans, the HIV-1 envelope glycoprotein gp120, or an anti-DC-SIGN antibody, which enabled real-time analysis of antigen internalization and trafficking.[68] QDs were internalized rapidly, accumulated in LAMP-1-positive lysosomes within ~30 min, and were retained within MHC II-positive intracellular compartments for up to 48 h. With increasing interest in the use of carbohydrate ligands as targeting motifs to deliver antigens to APCs, understanding antigen processing and trafficking dynamics may provide important insights for designing novel approaches to modulate immune cell responses or enhance antigen immunogenicity.

2.3.2 Mannose receptor targeting. Mannose receptor (MR) is another CLR that has been extensively studied for targeted delivery of antigens to APCs.[69,70,71] In the context of materials-based vaccines, MR-targeted delivery of polyanhydride (PA) particles has received significant attention because PA is biocompatible, biodegradable, and has inherent adjuvant properties.[72,73] Mannosylated PA nano- and microparticles were more efficiently internalized by DCs, upregulated DC expression of MR, and induced DC expression of the co-stimulatory molecules (CD86, MHC II and CD40) that are necessary to activate CD4+ T cells to induce an adaptive immune response.[74,75] Multivalent carbohydrate presentation by PA particles was necessary for DC maturation, since DC expression of co-stimulatory molecules was not up-regulated by soluble sugars. Similarly, PA nanoparticles modified with di-mannose and galactose bound to CLRs on alveolar macrophages, increased macrophage expression of MHC I/II, CD86, CD40, the CLR CIRE, and inflammatory cytokines, while also up-regulating expression of macrophage mannose receptor or macrophage galactose lectin, respectively.[76] Using an ocular immunization route, mannosylated PA nanoparticles loaded with the hot saline subcellular fraction extracted from *Brucella ovis* (*B. ovis)* conferred improved protection against *B. ovis* infection in mice when compared to a commercial vaccine.[77] More recently, mannose receptor targeting enhanced DC uptake of PA nanoparticles loaded with an HIV-1 antigen, while also up-regulating DC expression of the co-stimulatory surface marker CD40, and the CLR, CD206.[78]

In addition to PA, MR targeting has also been explored with other materials-based vaccines. For example, mannosylated PAMAM dendrimers enhanced antigen presentation, DC maturation, and antigen-specific CD4+ and CD8+ T cell responses in vivo.[79] Mannosylation of VLPs enhanced their uptake by murine and human DCs, while also increasing murine DC cross-presentation of a model MHC-I-restricted peptide antigen to antigen-specific CD8+ T cells in vitro.[80] Crosslink-stabilized, glucomannan-modified chitosan nanoparticles enhanced production of anti-bovine serum albumin IgG and IgA antibody production, as well as up-regulated expression of the inflammatory cytokines, IL-2 and IFN-γ, in murine oral immunization models.[81] Taken together, these examples highlight the enormous potential of MR targeting as a general strategy to enhance adaptive immunity via materials-based vaccines.

2.3.3 Dectin and DCIR targeting. One limitation of CLR targeting is that only a limited number of CLR-binding carbohydrate ligands have been identified to date.[82] Recently, Maglinao et al. developed a glycomaterial platform to identify new, selective CLR ligands based on microarrays displaying 52 biologically-relevant carbohydrates, including monosaccharides, disaccharides, sialylated oligosaccharides, sulfated saccharides, heparins, blood group antigens, high mannose structures, and tumor antigens, among others.[83] Fusion proteins of an Ig Fc-domain fragment and two copies of CRDs from CLRs of the Dectin-1 and dendritic cell immunoreceptor (DCIR) families were used to identify new glycan ligands with binding selectivity for Dectin-1 or DCIR. Conjugating glycan ligands identified via this screen to the model antigen OVA enhanced antigen uptake and presentation by DCs. Similar array-based approaches have proven useful for uncovering glycan-binding specificity of other lectins involved in innate and adaptive immunity,[84,85,86] suggesting that glycan arrays may be broadly useful for informing the design of new immunomodulatory materials or lectin-targeting delivery vehicles.

2.4 Targeting selectins to supress inflammation

P-, E-, and L-selectin, which are expressed by platelets, endothelial cells, and lymphocytes, mediate leukocyte and lymphocyte homing to sites of tissue damage during acute and chronic inflammation, as well as lymphocyte trafficking to secondary lymphoid organs, via their interaction with SLeX glycans.[87,88] Thus, inhibiting selectin-SLeX binding is a promising therapeutic target to suppress aberrant autoinflammatory responses or chronic inflammation. However, the affinity of selectins for monovalent SLeX ligands is low, in line with the general trend of lectin-carbohydrate interactions. To create an L-selectin inhibitor, Chaikof and colleagues developed multi-arm polyethylene oxide (PEO) star polymers terminated with a selectin-binding ligand, sulfated β-lactose.[89] 12-arm star polymer glycoclusters inhibited neutrophil and macrophage recruitment during thioglycollate-induced peritoneal inflammation to a similar extent as heparin, which inhibits L- and P-selectins via sulfate-dependent interactions.[89] In contrast to heparin, however, the star polymer selectively inhibited macrophage adhesion to immobilized L-selectin with an IC50 in the low nanomolar range. Notably, glycan valency was an important determinant of inhibitory activity, as 3- and 4-arm star polymers were ineffective for reducing neutrophil and macrophage recruitment during peritoneal inflammation. More recently, sulfated polyglycerol dendrimers and glycosylated polyglycerol dendrimers have demonstrated efficacy as inhibitors of both L- and P-selectin with IC50s in the nanomolar range,[90,91] while E-selectin inhibitors based on polyvalent SLeX glycomaterials have been developed using gold colloids and chitosan conjugates.[92,93] One notable challenge in the development of SLeX glycomaterials as E-selectin inhibitors is the inherent difficulty of SLeX synthesis and its susceptibility to enzymatic degradation. Thus, future efforts to create potent E-selectin inhibitors will likely benefit from improved synthesis methods or synthetic SLeX analogs with enhanced stability.

In addition to suppressing inflammation, selectin-binding glycomaterials can also be used to visualize inflammation during disease progression. For example, van Kasteren et al. developed iron oxide glyconanoparticles (GNPs) to image pre-symptomatic brain disease in vivo,[94] since many neurodegenerative disorders are initiated by leukocyte recruitment to the CNS and onset of local inflammation that induces tissue damage.[95,96,97] GNPs carrying millions of copies SLeX ("GNP-sLex") successfully crossed the blood-brain barrier of mice, localized to CNS capillaries overexpressing E- and P-selectin, and served as contrast agents that enhanced the resolution of MRI scans for neuroinflammation. Thus, glycomaterials engineered to target selectins may ultimately lead to 'theranostics' that can simultaneously monitor inflammatory disease progression and suppress aberrant or chronic inflammation.

3. Glycomaterials for adaptive immunotherapy

Many saccharide chemistries, glycan structures, and polysaccharide repeat sequences are exclusive to a particular pathogen or significantly altered during disease progression (e.g. malignant cell transformation),[98,99] and can be recognized by the immune system to distinguish self from non-self or altered self. For example, lipopolysaccharides found within the outer membrane of Gram-negative bacteria are recognized by TLR-4, a member of the TLR family of transmembrane "pathogen recognition receptors" (PRR) that can activate innate immune cells via binding to "pathogen associated molecular patterns", or PAMPs.[100] Similarly, collectins are a family of soluble PRR that can activate innate immune processes, such as the complement cascade, via binding to microbial glycan PAMPs.[101] Toward adaptive immunity, pathogen-specific carbohydrates can be effective as antigens for prophylactic vaccines that confer protection against infection by various bacteria, including *Neisseria meningitidis*, *Streptococcus pneumoniae*, *Haemophilus influenza* type b (Hib), and *Salmonella typhi*.[102] Significant efforts are now devoted to the use of carbohydrate antigens to develop prophylactic vaccines to prevent viral infection, as well as anti-cancer immunotherapies.[103]

Unfortunately, carbohydrates are typically poor immunogens, which greatly challenges efforts to develop effective anti-carbohydrate vaccines. In part, this is because B cell production of anti-carbohydrate antibodies is typically a "T-independent" process, occurring independently of B cell recruitment of CD4+ T cell "help" via presentation of peptide antigens on MHC II.[104] As a result, interactions between T and B cells in germinal centers that can lead to production of robust and persistent antigen-specific antibodies, such as antibody affinity maturation, IgM-to-IgG class switch recombination, and B cell differentiation into memory cells, do not occur.[105] Instead, BCR recognition of carbohydrate antigens typically induces clonal expansion of short-lived B cells that produce anti-carbohydrate IgM antibodies with low antigen-binding affinity.[106]

Avery and Goedel pioneered early efforts to increase carbohydrate immunogenicity in 1931, by conjugating carbohydrate antigens onto a highly immunogenic 'carrier protein', which can be processed and loaded into MHC II to recruit CD4+ T cell help.[107] Several clinically successful vaccines have been developed using this approach to date,[108] although carrier proteins are not without their own challenges. In particular, the chemical activation procedures and protein-carbohydrate linkers used often provide heterogeneous glycan constructs, yet valency, density, and carbohydrate conjugation site can be important determinants of immunogenicity, as discussed in more detail below. In addition, antibodies are often directed towards the carrier protein and linker, rather than the carbohydrate epitope, which can lead to antigen suppression.[109,110] In light of these limitations, there has been increasing interest in the design of self-adjuvanting monovalent glycoantigens as vaccines, and we direct the reader to excellent recent reviews on this topic.[111,112] In addition, synthetic materials are receiving significant attention

as alternatives to carrier proteins for carbohydrate vaccines because they can provide multivalent antigen display on a background that is well defined, reproducible, modular, and minimally immunogenic. In the following sections, we highlight recent advances in combining carbohydrates and synthetic materials to create more effective glycovaccines.

3.1 Glycovaccines as cancer immunotherapies

Glycovaccines are intended to educate the immune system to recognize changes in glycosylation patterns that are unique to a disease state. For example, certain mucin-like glycoproteins, such as MUC1, are over-expressed by tumor cells.[113] The apical-basal distribution pattern of MUC1 and other glycoproteins can also differ on tumor epithelial cells when compared to healthy cells.[114] Tumor cell surfaces are also often decorated with glycans that are rare in healthy tissues (e.g. Tn antigen), due to genetic mutations of glycosyltransferases and glycosidases, as well as transcriptional or translational changes in their expression.[115] As a result, there is increasing interest in glycovaccines that present tumor-associated carbohydrate antigens, or "TACAs", to the immune system to establish tumor-specific immunity. However, TACA glycovaccines are often hindered by the weak, T-independent immunogenicity of carbohydrate antigens. Previous efforts to create T-dependent TACA vaccines via carrier proteins have been largely unsuccessful due to the strong immunogenicity of the carrier protein or linker, which ultimately leads to TACA epitope suppression.[116,117] Thus, glycovaccines that can elicit robust T-dependent immune responses to TACA, while eliminating or greatly reducing carrier immunogenicity, are receiving increasing attention. In the following sections, we highlight recent advances in using synthetic materials to create TACA glycovaccines that can meet these requirements.

3.1.1 Liposomal TACA vaccines. Liposomal formulations are advantageous for vaccine development because they are easy to prepare and can provide a platform for multivalent antigen display that is itself largely non-immunogenic.[118,119] Boons et al created liposomal TACA vaccines based on lipidated glycopeptides that integrate into small unilamellar vesicles (SUV).[120] In an early iteration, they synthesized a three-component molecule comprising: 1) *Tn antigen*; 2) *the YAF peptide*, an MHC II-restricted T helper cell epitope peptide*;* and 3) *Pam_3Cys*, a TLR-2 agonist that can act as an adjuvant to activate APC, while also enabling incorporation of the glycopeptide into SUV for multivalent display. In murine models, SUV with integrated lipidated glycopeptides produced high titers of IgG antibodies that were specific for the Tn antigen, without inducing antibodies against the YAF peptide, the latter demonstrating that the carrier fulfills the requirement of being minimally immunogenic. Notably, previous efforts to generate TACA immunity by conjugating a carbohydrate antigen directly to Pam_3Cys yielded no or low IgG antibody titers, highlighting the importance of the YAF peptide for providing T cell help to induce robust antibody production and class switch recombination.[121,122]

The fully-synthetic nature of lipidated glycopeptides enables modular construction of compounds with various integrated adjuvants, which has provided insights into the importance of adjuvant selection in glycovaccine design. For example, lipidated MUC1 glycopeptides bearing the adjuvant Pam_3CysSK_4, which engages TLR-1 and -2, elicited higher titers of anti-MUC1 IgG antibodies than glycopeptides bearing Pam_2CysSK_4, which engages TLR-2 and -6.[123] Similarly, replacing Pam_3Cys with lipidated amino acids that are unable to bind TLRs greatly reduced anti-MUC1 IgG titers.[124] Notably, immunization with an external adjuvant (e.g. Pam_3Cys or monophosphoryl lipid A) and liposomes of MUC1 glycopeptides with lipidated amino acids increased anti-MUC1 IgG antibody titers, however these antibodies bound antigen-expressing cancer cells weakly when compared to antibodies raised via immunization with MUC1 glycopeptides bearing Pam_3Cys.[124] Similarly, low titers of anti-MUC1 IgG antibodies were raised when the antigen, T cell epitope, and adjuvant were formulated into liposomes as individual components, suggesting that covalent conjugation of an antigen and T cell epitope may be necessary for concurrent processing and presentation of B and T cell epitopes by the same APC to potentiate TACA immunity.

The fully-synthetic nature of lipidated glycopeptides also enabled design of a glycovaccine that can provide combined humoral and cellular immunity, which is advantageous for providing broad-spectrum anti-tumor immune responses.[125] In particular, Boons et al. created a lipidated glycopeptide having a MUC1-derived glycopeptide antigen that can be presented by MHC class I to activate antigen-specific cytotoxic T lymphocytes (CTLs) (Figure 3a). Immunization with lipidated glycopeptide liposomes significantly reduced tumor burden in mice challenged with MUC1-bearing tumor cells when compared to mice immunized with liposomes of a non-glycosylated antigen or a mixture of adjuvant and glycopeptide antigen (Figure 3b). The enhanced anti-tumor immune responses provided by lipidated glycopeptide liposomes were due to increased anti-MUC1 total IgG antibody titers, including increased anti-carbohydrate IgG3 titers,[126] as well as induction of CD8+ CTLs that recognize both glycosylated and non-glycosylated MUC1-derived peptides (Figure 3c). Together, these observations suggest that proper selection of antigens, T helper epitopes, and adjuvants can be used to tailor the immune system response to TACA vaccines to enhance anti-tumor immunity.

3.1.2 Supramolecular TACA vaccines. The self-assembly of individual molecules into supramolecular nano-scale architectures, such as nanoparticles and nanofibers, can provide highly-ordered platforms for multivalent display of molecules. As a result, there is growing interest in the use of supramolecular assemblies of amino acid-based molecules as vaccines.[127] The following sections will highlight recent developments in glycovaccines based on VLPs with external adjuvants, nanoparticles with integrated adjuvants, and self-adjuvanting peptide nanofibers.

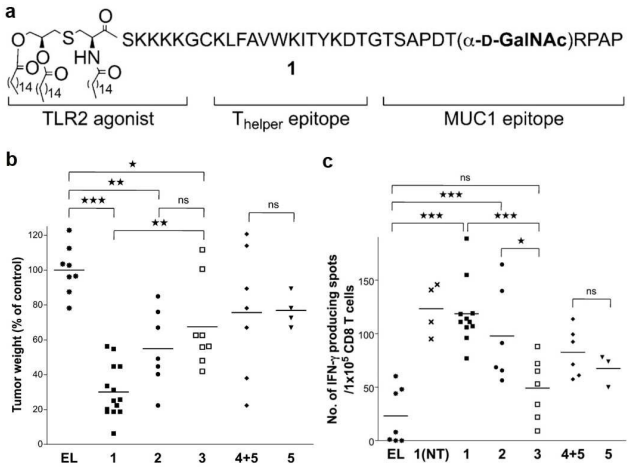

Figure 3: Lipidated glycopeptide that raises TACA immunity reduces tumor burden. A) Schematic of a lipidated MUC1 glycopeptide. B) Lipidated glycopeptide vaccines reduced mammary tumor burden in MUC1.Tg mice. C) IFN-γ producing CD8+ T cells in MUC1.Tg mice. EL = empty liposomes, 1 = liposomes containing lipidated MUC1 glycopeptide in (a). Reproduced with permission from [125].

3.1.2a Virus-like particles. Huang, Finn, and colleagues have extensively investigated VLPs as supramolecular carriers for TACA vaccines. VLPs are particularly advantageous because they often contain multiple T helper epitopes and are assembled from highly repetitive protein units that form an exquisitely organized surface for multivalent antigen display. In an initial demonstration, a VLP glycovaccine carrying ~60 copies of Tn antigen conjugated to an engineered cysteine-mutant of cowpea mosaic virus (CPMV) raised high titers of anti-Tn IgG antibodies that bound to Tn-expressing MCF-7 breast cancer cells and multi-drug resistant NCI-ADR RES breast cancer cells when co-administered with an external adjuvant (complete Freund's adjuvant (CFA)).[128] Subsequently, a VLP glycovaccine carrying >2000 copies of Tn antigen conjugated to tobacco mosaic virus (TMV) induced robust anti-Tn antibody production when co-administered with CFA.[129] Notably, the site of Tn antigen conjugation onto TMV was an important determinant of immunogenicity, since VLP with Tn conjugated to Tyr 139 elicited high anti-Tn IgM and IgG antibody titers, while Tn conjugated to the N-terminus failed to induce anti-Tn antibodies.

More recently, Huang and Finn created a VLP glycovaccine carrying a maximum of 340 copies of Tn antigen conjugated to Qβ bacteriophage capsids, in which antigen dose could be precisely varied by altering the conditions of Tn conjugation via Huisgen 'click' cycloaddition.[130] Qβ-Tn glycovaccines also produced high anti-Tn antibody titers when co-administered with CFA, and were significantly more potent than the CMV-Tn and TMV-Tn vaccines described above. The latter was particularly noteworthy due to the greatly reduced antigen load on Qβ-Tn when compared TMV-Tn (~300 vs ~2000), and demonstrates that antigen valency alone does not dictate immunogenicity. However, antigen density governed antibody class switch recombination. In particular, Qβ VLP having high Tn antigen density produced significantly higher IgG titers than VLP with 'medium' or 'low' antigen density, while IgM titers were similar in all groups. Thus, the highly reproducible control of antigen valency and density on VLP can be harnessed to elicit optimal anti-carbohydrate immune responses, and may thereby address limitations of conventional carrier vaccines having heterogeneous glycan composition and organization.

In a subsequent study, however, Huang, Finn, and colleagues noted that in addition to high anti-Tn IgG titers, Qβ-Tn rapidly induced high titers of IgG against the non-natural triazole linker, which suppressed anti-Tn responses.[131] Replacing the triazole linker with an alkyl amide greatly increased anti-Tn IgG titers, while also expanding the breadth of the raised antibody repertoire. As a result, vaccination with Qβ-alkyl amide-Tn in combination with chemotherapy protected mice from cancer to a much greater extent than vaccination or chemotherapy alone. Thus, this work provides an important example that linker chemistry is important for avoiding antigen suppression by glycomaterial vaccines, a noted limitation of conventional carrier protein vaccines.

Taken together, these examples highlight the potential of VLPs as TACA vaccine carriers, and begin to establish key design considerations for further improving their efficacy. In particular, the reproducible, site-specific conjugation of glycoantigens to VLP, in addition to fine control of antigen load and density, may address limitations of conventional carrier vaccines. It is worth noting, however, that the VLP-TACA vaccines described above were administered with the external adjuvant, CFA, which is potentially toxic and of limited clinical relevance.[132] For other platforms, such as lipidated glycopeptides and regioselectively addressable functionalized templates (RAFT), covalent conjugation of palmitic acid (Pam) to TACA provided "self-adjuvanting" glycovaccines, which eliminated the need for potentially deleterious external adjuvants.[133,134,135] Moving forward, it may be of interest to further modify VLP-TACA vaccine surfaces with Pam, or other molecular adjuvants, to render them self-adjuvanting.

3.1.2b Self-assembled nanoparticles with integrated adjuvants. Supramolecular assemblies with integrated adjuvants are also gaining interest as glycovaccines, since they will likely have improved safety profiles and increased clinical relevance when compared to vaccines requiring external adjuvants. Toward this end, Payne and colleagues created glycovaccines with an integrated adjuvant via self-assembling tri-component glycopeptides.[136] In particular, they synthesized molecules comprising: 1) a $Pam_3CysSer$ adjuvant, 2) a universal T helper epitope, PADRE; and 3) a MUC1 peptide antigen bearing (a) no glycans, (b) 5 copies of Tn antigen, or (c) 5 copies of T antigen. Each peptide or glycopeptide molecule self-assembled into nanoparticles with diameters of ~17-25 nm under aqueous conditions. Intradermal delivery of peptide or glycopeptide nanoparticles raised IgG antibodies that recognized the respective MUC1 peptide or glycopeptide antigen presented on the surface of the nanoparticle. The raised antibodies also bound MCF-7 breast cancer cells and

B16 melanoma cells transfected with the MUC1 gene, suggesting their potential as anti-cancer vaccines.

Alternatively, Li and colleagues recently developed a multi-layer approach to create a TACA glycovaccine with an integrated adjuvanting molecule. In a three-step process: 1) a T helper epitope having an oligo(lysine) tail self-assembled into positively-charged spheres; 2) spheres were coated with the negatively-charged adjuvant, γ-polyglutamic acid; and 3) a positively-charged MUC1 glycopeptide antigen was electrostatically complexed to the spheres. Intraperitoneal injection of spheres raised anti-MUC1 antibodies that were predominantly IgM and IgG2a isotypes, with lower titers of IgG1 and IgG3 antibodies. The raised antibodies bound MCF-7 cancer cells and activated complement dependent cytotoxicity in vitro. Together, these results suggest that self-assembled multilayers of an adjuvant, antigen, and T helper epitope may provide the basis for efficacious anti-cancer vaccines.

3.1.2c Self-adjuvanting peptide nanofibers. Synthetic peptides that self-assemble into β-sheet nanofibers can act as vaccines that induce robust humoral and cellular immunity to peptide and protein antigens without the need for external adjuvants, via minimally inflammatory mechanisms that are still largely undefined.[137,138,139,140,141,142] In the context of glycovaccines, Li and colleagues demonstrated that self-assembling glycopeptide nanofibers can also act as self-adjuvating vaccines to generate TACA immunity.[143] In particular, variants of the β-sheet fibrillizing peptide Q11 terminated with MUC1 glycopeptide antigens self-assembled into nanofibers that elicited anti-MUC1 antibodies. Glycosylation site was important, with antigens having Tn conjugated to Thr 9 of MUC1 raising higher IgG titers than a non-glycosylated antigen or antigen glycosylated only at Thr 16. Antibodies raised against MUC1 glycosylated at Thr 9/Thr 16 mediated complement-dependent cytotoxicity of MCF-7 cancer cells, suggesting the potential of self-assembled glycopeptide nanofibers as effective anti-cancer vaccines. Notably, IgG2a and IgM were the predominant antibody isotypes raised, which was not surprising given that the nanofiber vaccine lacked a T helper epitope that is likely required for robust class switch recombination. Recently, Collier and colleagues demonstrated that multicomponent nanofibers consisting of co-assembled Q11 variants terminated with a peptide B cell antigen or universal T helper epitope can enhance production of antibodies against the B cell antigen,[144] which may also be useful for enhancing the immunogenicity of TACA antigens.

3.1.3 Emerging materials-based glycovaccines. In addition to liposomes and supramolecular assemblies, various other synthetic materials are receiving attention for TACA vaccines. For example, Kunz et al. created a TACA vaccine from an otherwise bio-inert, water-soluble poly(N-(2-hydroxypropyl)methacrylamide (HPMA) polymer modified with a MUC1 glycopeptide antigen and T helper epitope.[145] Huang et al. demonstrated that water-soluble block co-polymers modified with TACA can raise robust anti-Tn IgG antibodies.[146] Notably, low, nearly equivalent titers of anti-polymer IgG antibodies were raised against both the glycopolymer and a non-glycosylated control, demonstrating that the carrier fulfills the important requirement of being minimally immunogenic.[146]

Entanglement of linear polymer-glycoantigen conjugates may render some antigen molecules inaccessible for recognition by B cells, thereby diminishing the adaptive immune response. To address this physical limitation, Kunz et al. recently developed a hyperbranched polymer vaccine for multivalent glycopeptide antigen display.[147] They proposed that the dendrimer-like architecture of the hyperbranched polymer would provide multivalent antigen display on the "surface" of a globular vehicle, while the water-soluble nature of the dendrimer arms would afford antigen flexibility. Together, these features are expected to enhance antigen availability for recognition by the immune system. Mice immunized with hyperbranched polymers bearing T helper epitope-MUC1 glycopeptides in CFA produced high titers of anti-MUC1 IgG antibodies that bound to MUC1-expressing cancer cells in vitro, providing initial proof-of-concept for hyperbranched polymers as carriers for TACA vaccines.

Inorganic nanoparticles based on gold or metal oxides are of increasing general interest as materials for biomedical applications because they are easily prepared with a broad range of sizes, are nontoxic and biocompatible, and provide significant multivalency due to their high surface-to-volume ratio.[148] In the context of glycovaccines, Barchi and colleagues demonstrated that gold nanoparticles decorated with various thiol-terminated MUC4-derived peptides bearing Thomsen-Friedenreich (TF) glycoantigens and a thiol-terminated adjuvant peptide derived from complement Cb3 produced antigen-specific IgM and IgG antibodies.[149] Similar to MUC1-Q11 nanofibers discussed above,[143] the magnitude and quality of the immune response to these glyconanoparticles was dependent on the site of TF glycoantigen conjugation to the MUC4 peptide, with a variant having a single copy of TF antigen conjugated to a threonine at the 10th position eliciting the most robust antibody isotype switching from IgM to IgG. In an alternative approach, Cameron and colleagues demonstrated that a thiolated polymer of Tn antigen adsorbed onto gold nanoparticles produced higher titers of anti-Tn IgG antibodies that were more reactive towards mucin proteins displaying Tn than antibodies raised by soluble polymer.[150] More recently, Sungsuwan et al. demonstrated that iron oxide nanoparticles modified with MUC1 lipo(glyco)peptides raised MUC1-specific antibodies that recognized cancer cells, and induced complement-mediated cancer cell cytotoxicity.[151]

Taken together, the above examples highlight the enormous potential of synthetic glycomaterials for addressing the limitations of conventional protein conjugate glycovaccines as cancer immunotherapies. In particular, vaccines based on synthetic materials can often be designed to incorporate the minimum number of domains needed to induce robust, long-lasting immunity against otherwise poorly immunogenic antigens. In addition, the chemical nature of the antigens, as well as their physical display within the scaffold, can often be independently tailored to optimize the resulting immune

response. Moving forward, the compositional flexibility provided by synthetic glycomaterial vaccines is likely to enable increasing efficacy through systematic redesign.

3.2 Glycomaterials as diagnostics of anti-carbohydrate immunity

Through the normal course of immune surveillance, the host immune system can raise antibodies that are reactive towards disease-related glycoantigens, and detection of these antibodies can be useful for diagnosis of disease onset and progression. For example, Globo-H is a TACA that is over-expressed in breast cancer.[152] Many breast cancer patients raise anti-Globo-H autoantibodies, which are therefore gaining interest as disease-specific biomarkers. Wang et al. developed a microarray displaying different densities of Globo-H antigens and other truncated analogs that can detect differences in anti-Globo-H levels in sera of healthy patients and those with breast cancer.[153] Notably, this platform was able to detect antibodies at the attomole level, making it 5x more sensitive than conventional enzyme-linked immunosorbent assay (ELISA). More recently, Yu and colleagues demonstrated that glycan microarrays can be used to detect changes in patient serum glycan antibodies as an early biomarker for hepatocellular carcinoma.[154] In addition to cancer glycoantigens, patients with autoimmune diseases, such as Crohn's disease,[155] rheumatoid arthritis,[156] and multiple sclerosis[157] often develop anti-glycan antibodies that can serve as unique disease-specific biomarkers that are detectable with glycan microarrays.

3.3 Glycomaterials for glycoantigen design

The humoral immune systems of some HIV-infected individuals can naturally raise antibodies that broadly neutralize diverse HIV strains.[158] Often, these 'broadly neutralizing antibodies' (bnAb) recognize glycoantigens of the gp120 coat protein that are highly conserved,[159] which has led to increasing interest in glycovaccines for HIV infection prophylaxis.[160] Toward this end, there are significant ongoing efforts to identify HIV glycan architectures that are recognized by bnAbs to inform the design of glycoantigens, and glycomaterials are gaining attention in this regard. For example, Davis and colleagues demonstrated that VLP bearing D-fructose monosaccharides bound bnAb *2G12,* which binds the D1 arm of terminal mannosides, with higher affinity than mannosylated VLPs.[161] In addition, fructosylated-VLPs elicited higher titers of anti-D1 arm antibodies than mannosylated-VLPs, however the raised antibodies failed to neutralize HIV.[161] More recently, Krauss and colleagues used *Selection with Modified Aptamers*, or "SELMA", for the directed evolution of DNA-based oligomannose glycoclusters with low nanomolar to picomolar affinity for 2G12.[162,163,164] Alternatively, Penadés and colleagues recently demonstrated that gold nanoparticles decorated with the tetramannoside D1 arm and pentamannose D2/D3 arms of HIV gp120 $Man_9GlcNAc_2$ antennas had nearly 4-fold higher affinity for 2G12 HIV bnAb when compared to nanoparticles bearing the tetramannoside alone, which approaches the affinity of 2G12 for Man_9 oligosaccharides.[165] Thus, glycomaterials with modular and tunable carbohydrate composition may eventually prove useful for identifying glycoantigens for raising bnAb, or provide effective prophylactic HIV vaccines.

4. Glycomaterials for non-adaptive infection prophylaxis

In addition to their role as antigens that can be recognized by the host immune system, carbohydrates often mediate early events in pathogen infection via their interactions with carbohydrate-binding proteins. For example, type 1 fimbriae extending from the surface of *E. coli* are terminated by lectins that mediate bacterial adhesion to host glycans.[166] Bacterial AB5 toxins, including cholera toxin, *E. coli* heat-labile toxin, and Shiga-like toxin, are composed of a toxic A subunit that is associated with a carbohydrate-binding B-pentamer, the latter being necessary for toxin entry into host cells.[167] As a result, glyco-based therapeutics capable of disrupting these protein-carbohydrate interactions are gaining increased interest as alternatives to glycovaccines for pathogenic infection prophylaxis. Multivalent glycomaterials that can inhibit bacterial adhesion, biofilm formation, and the action of bacterial toxins have been the topic of many excellent recent reviews,[168,169,170,171] and will therefore not be discussed in detail here. Instead, this section will highlight recent advances in glycomaterials designed to inhibit viral pathogen entry into host immune cells by interfering with interactions between host lectins and pathogen glycans.

4.1 Viral entry via host lectins

In many instances, binding of host lectins to non-self carbohydrates acts as a "danger signal" that informs the immune system of the presence of a foreign entity. For example, opsonization of mannosylated pathogens by host mannose-binding lectin can activate the lectin pathway of the complement cascade to eradicate bacterial and fungal pathogens.[172] In addition, the preceding section presented vaccines that can educate the immune system to recognize foreign carbohydrate epitopes by raising carbohydrate-binding antibodies. However, certain viral pathogens have evolved enhanced infectivity through interactions with host lectins. One such example is the infection of immature DCs residing at mucosal sites by HIV, Ebola, or Dengue through DC-SIGN receptor binding to high mannose glycans on viral envelope glycoproteins.[173] Once infected, these DCs can then traffic to lymph nodes, where they efficiently transfer virions to T lymphocytes to facilitate viral replication.[174] In addition, the bivalent host lectin, galectin-1, can increase HIV infectivity by crosslinking viral and host glycoproteins.[32,175] Thus, interfering with viral glycoprotein binding to host lectins is a promising target for viral infection prophylaxis and anti-viral therapeutics.

Drugs that can interfere with protein-carbohydrate interactions have been pursued for nearly 40 years, however their therapeutic efficacy was long hindered by the low binding

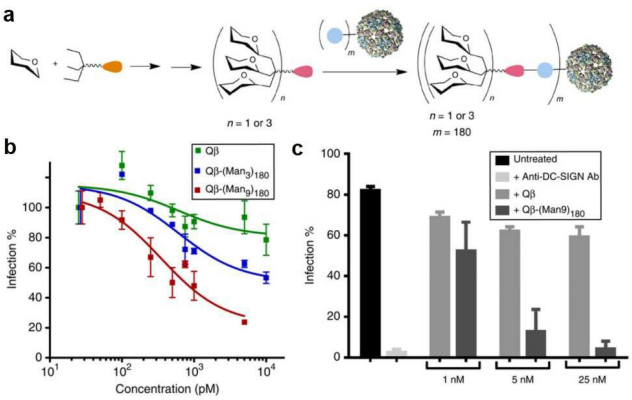

Figure 4: Virus-like glycodendronanoparticles inhibit Ebola infection via DC-SIGN. A) Schematic of mannosylated virus-like glycodendronanoparticles. B) Infection rates of T cells expressing DC-SIGN by EboGP pseudovirus in the presence of VLP and mannosylated virus-like glycodendronanoparticles. C) Inhibition of human DC *cis* infection by EboG via mannosylated virus-like glycodendronanoparticles. Reproduced with permission from [179].

affinity of small molecules and monovalent carbohydrates for highly-hydrated lectin CRDs.[176] Cecioni et al. recently compiled a survey comparing mono- and multivalent lectin inhibitors, which highlights the importance of the glycocluster effect in lectin inhibitor design.[28] The following sections will highlight recent advances in synthetic multivalent glycomaterials to inhibit viral entry via the host lectin, DC-SIGN.

4.2 Inhibitors of Ebola entry

Toward the design of effective multivalent Ebola infection inhibitors, Rojo, Delgado, and colleagues demonstrated in the early 2000s that hyperbranched Boltorn™ dendritic polymers bearing 32 terminal mannose residues inhibited Ebola viral envelope *cis* infection of Jurkat T cells, as well as *trans* infection via DC-SIGN expressing cells, with a micromolar IC50.[177] Replacing mannose ligands with 'pseudomannoside' glycomimetics significantly improved the efficacy of glycodendrimers as Ebola entry inhibitors, decreasing the IC50 to the nanomolar range.[178] Conjugating glycodendrimers to VLP provided glyconanoparticles with up to 1620 pendant mannose ligands (Figure 4a), which inhibited Ebola entry via DC-SIGN into T cells (Figure 4b) and DCs (Figure 4c) with an IC50 in the picomolar range, highlighting the importance of multivalency for inhibitor efficacy.[179] More recently, mannosylated fullerenes were explored as an alternative to VLP-based nanoparticles for inhibition of Ebola entry via DC-SIGN.[180] Interestingly, glycofullerenes with 36 mannose residues attached via a short linker-arm were less effective inhibitors than those with 12 mannose residues attached via the same linker-arm. However, glycofullerenes with 36 mannose residues attached via a longer linker-arm were more effective than either short linker-arm formulation. Together, these observations suggest that ligand availability and flexibility, in addition to valency, are important factors that should be considered in the design of glycomaterials as viral entry inhibitors.

4.3 Inhibitors of HIV entry

Multivalent glycomaterials are also receiving attention as inhibitors of HIV infection via DC-SIGN. In an early example, Penadés and colleagues created gold nanoparticles (AuNP) modified with thiolated mannose oligosaccharides, which inhibited binding of the HIV coat protein gp120 to DC-SIGN with a nanomolar IC50.[181] Subsequently, they demonstrated that AuNP modified with a thiolated linear mannose tetrasaccharide, or branched mannose penta- and heptasaccharides, inhibited HIV *trans* infection of human T cells by preventing viral entry into human activated peripheral blood mononuclear cells via DC-SIGN.[182] Around this same time, Rojo, Bernardi, and colleagues demonstrated that tetravalent Boltorn™ dendrons terminated with pseudomannoside glycomimetics effectively inhibited HIV *trans* infection of CD4+ T cells via DC-SIGN expressing THP-1 cells in vitro.[183] Importantly, these pseudomannoside dendrimers also prevented HIV infection of explanted human cervical tissues under conditions that mimic compromised epithelial integrity, suggesting their potential for clinical use.[184] Using a library of glycodendrimers with different valencies and terminal glycans or glycomimetics, Varga et al. identified a hexavalent bis-benzamide pseudo-disaccharide dendrimer capable of inhibiting HIV *trans* infection of T cells, as well as DC-SIGN mediated uptake of Dengue virus, with a micromolar IC50.[185] More recently, Ordanini et al. demonstrated that trimers of the same bis-benzamide pseudo-disaccharide linked by a rod-like, phenylene-ethylene "rigid spacer" provided even more potent inhibitors of HIV *trans* infection, having a nanomolar IC50, likely due to chelation and statistical rebinding.[186] Importantly, the bis-benzamide pseudo-disaccharide integrated into these inhibitors is highly selective for binding to DC-SIGN versus Langerin,[187] a CLR expressed by Langerhans DCs that inhibits HIV infection by internalizing, sequestering, and degrading the virus.[188] Thus, glycodendrimers that selectively inhibit the activity of DC-SIGN may work in concert with natural Langerin-mediated mechanisms of HIV infection inhibition, rather than against them.

In an alternative design, Wagner and colleagues created glycolipids with a monomeric mannose headgroup that inhibited HIV *trans* infection with micromolar IC50s.[189] Replacing the monomeric mannose headgroup with a trimeric mannose ligand further improved the efficacy of glycolipids as inhibitors of HIV *trans* infection.[190] Interestingly, trimannoside glycolipids self-assembled into micelles were more potent HIV entry inhibitors than single molecules or covalently-crosslinked glycolipid polymers, suggesting an important correlation between micelle dynamics and inhibitor binding to DC-SIGN.[190]

Taken together, these examples demonstrate that multivalent glycomaterials can inhibit viral *cis* infection and *trans* infection via DC-SIGN. Notably, although expected correlations between ligand valency and binding affinity are often apparent, it is becoming increasingly more evident that glycan spatial orientation and availability are also important features of viral entry inhibitor design.

Summary

Carbohydrates are integral to innate and adaptive immune system function, and are receiving increasing interest as targets and bioactive components of therapeutics that can leverage the immune system to treat current pathologies and prevent onset of future disease. There is enormous diversity in carbohydrates found throughout nature, and their immunological activity is often related to their specific, non-covalent binding to a particular protein (e.g. lectins, antibodies, and BCRs). Elucidating details of the 'sugar code' relating carbohydrate-protein binding specificity has greatly accelerated efforts to create therapeutics that can activate, attenuate, or inhibit immunological processes by promoting, mimicking, or disrupting specific protein-carbohydrate binding events. Equally important, however, is recognizing that multivalent display of immobilized glycans throughout nature (e.g. on the cell membrane or extracellular matrix) can greatly enhance the otherwise weak affinity of monovalent protein-carbohydrate interactions, while also establishing steric constraints that dictate protein-carbohydrate binding specificity. Synthetic materials with dense, highly repetitive molecular architectures, such as lipids, polymers, supramolecular assemblies, and nanoparticles, are ideally suited to recapitulate the avidity effects of multivalent "glycoclusters" prevalent in nature. As a result, synthetic multivalent glycomaterials can provide therapeutics that more effectively modulate the behavior of innate and adaptive immune cells via lectin binding, enhance the efficacy of vaccines that confer adaptive recognition and memory of non-self or 'altered self' carbohydrate antigens, and potently inhibit viral entry into host immune cells. Nonetheless, many challenges remain. In particular, it is becoming increasingly more apparent that glycan spatial organization and steric availability may be as important as carbohydrate chemistry and valency for optimizing protein binding affinity and specificity. In addition, the role of many carbohydrates and their cognate proteins in particular disease states remain largely unknown. Multivalent glycomaterials are uniquely positioned to elucidate physicochemical aspects of glycoclusters that confer maximal protein-carbohydrate binding affinity and specificity, while also providing robust tools for high-throughput interrogation of ill-defined protein-carbohydrate binding events. As a result, glycomaterials hold enormous promise for developing new immunotherapies for pressing diseases, such as cancer and autoimmunity, as well as prophylactics for HIV and other emerging pathogens.

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Abbreviations

APCs	Antigen-presenting cells
BCR	B cell receptor
CLR	C-type lectin receptor
CRD	Carbohydrate recognition domain
CTL	Cytotoxic T Lymphocyte
CX	Complex-type
DC	Dendritic cell
DCIR	Dendritic cell immunoreceptor
DC-SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
GlcNAc	N-Acetylglucosamine
GNP	Glyconanoparticle
Ig	Immunoglobulin
LacNAc	N-Acetyllactosamine
MHC I and II	Major histocompatibility complex I and II
MPLA	Monophosphoryl lipid A
MR	Mannose receptor
MUC1	Mucin-like glycoprotein
PAMPs	Pathogen associated molecular patterns
PRR	Pathogen recognition receptor
Siglecs	Sialic acid-binding immunoglobulin-type lectins
SLeX	Sialyl Lewis X
TACA	Tumor-associated carbohydrate antigen
TLR	Toll-like receptor
VLP	Virus-like particle

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