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Research review paper

Glycovaccinology: The design and engineering of carbohydrate-based vaccine components

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ABSTRACT

Vaccines remain one of the most important pillars in preventative medicine, providing protection against a wide array of diseases by inducing humoral and/or cellular immunity. Of the many possible candidate antigens for subunit vaccine development, carbohydrates are particularly appealing because of their ubiquitous presence on the surface of all living cells, viruses, and parasites as well as their known interactions with both innate and adaptive immune cells. Indeed, several licensed vaccines leverage bacterial cell-surface carbohydrates as antigens for inducing antigen-specific plasma cells secreting protective antibodies and the development of memory T and B cells. Carbohydrates have also garnered attention in other aspects of vaccine development, for example, as adjuvants that enhance the immune response by either activating innate immune responses or targeting specific immune cells. Additionally, carbohydrates can function as immunomodulators that dampen undesired humoral immune responses to entire protein antigens or specific, conserved regions on antigenic proteins. In this review, we highlight how the interplay between carbohydrates and the adaptive and innate arms of the immune response is guiding the development of glycans as vaccine components that act as antigens, adjuvants, and immunomodulators. We also discuss how advances in the field of synthetic glycobiology are enabling the design, engineering, and production of this new generation of carbohydrate-containing vaccine formulations with the potential to prevent infectious diseases, malignancies, and complex immune disorders.

1. Introduction

Carbohydrates are ubiquitous in nature and are a vital component of all living organisms. Simple and complex carbohydrates (also known as glycans) play important functional roles in myriad life processes including cell growth and development, cell-cell communication, and immune recognition/response, among others (Varki, 2023; Varki, 1993, 2017). In eukaryotes, glycans feature prominently on the cell surface, primarily occurring as conjugates to proteins, lipids, and nucleic acids. This ensemble of different cell-surface glycoforms is organized in a polymeric meshwork called the glycocalyx. From a physical perspective, the glycocalyx is a "slime" layer that maintains membrane integrity and protects cells from chemical and mechanical stresses or foreign invaders (Chin-Hun Kuo et al., 2018). However, the glycocalyx has also evolved

Abbreviations: APC, antigen-presenting cell; BCR, B cell receptor; CFGpS, cell-free glycoprotein synthesis; CPS, capsular polysaccharide; CLR, C-type lectin receptor; DC, dendritic cell; DT, diptheria toxoid; EPS, exopolysaccharide; GBS, Group B *Streptococcus*; Hib, *Haemophilus influenzae* type b; IgG, immunoglobulin G; IgM, immunoglobulin M; glycOMV, glycosylated outer membrane vesicle; LOS, lipooligosaccharide; LPS, lipopolysaccharide; MHC, major histocompatibility complex; MOG, myelin oligodendrocyte glycoprotein; NLR, Nod-like receptor; O-PS, O-antigen polysaccharide; OVA, ovalbumin; PAMP, pathogen-associated molecular pattern; PRR, pattern recognition receptor; PG, peptidoglycan; Siglec, sialic-acid binding immunoglobulin-type lectins; TA, teichoic acid; TACA, tumor-associated carbohydrate antigen; TLR, Toll-like receptor; Treg, regulatory T cells; TT, tetanus toxoid; ZPS, zwitterionic polysaccharides.

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more advanced functionality. For example, it acts as a structural scaffold that spatially organizes cell surface receptors, multimolecular signaling complexes, and lipid microdomains (Ostrowski et al., 2016). It also provides a distinctive cellular code that influences innate and adaptive immune responses to pathogens (Rabinovich et al., 2012) and controls growth and progression of cancer (Pinho and Reis, 2015).

In bacteria, the glycocalyx similarly involves a dense network of important glycan structures that coat the cell surface. Interestingly, whereas eukaryotic glycans are restricted to approximately 10 monosaccharide building blocks that are assembled in varying numbers and combinations, prokaryotic organisms synthesize a much wider range of carbohydrate structures, including pseudaminic acid and legionaminic acid (Morrison and Imperiali, 2014), that are distinct from those commonly found in eukaryotes. In gram-negative bacteria, the cell surface is comprised of a thin layer of peptidoglycan (PG) that is surrounded by an outer membrane containing lipopolysaccharide (LPS) and lipooligosaccharide (LOS) (Raetz and Whitfield, 2002; Tytgat and Lebeer, 2014; Whitfield et al., 2020). gram-positive bacteria lack LPS and LOS and are instead surrounded by a thicker PG layer with embedded glycosylated teichoic acids (TAs). Both gram-negative and -positive species produce an array of other structurally diverse cell-surface glycoconjugates including capsular polysaccharide (CPS), exopolysaccharide (EPS), and glycosylated proteins such as surface layer (S-layer) glycoproteins, flagellin, pilin, and others (Benz and Schmidt, 2002; Tytgat and Lebeer, 2014). Among gram-positive bacteria, mycobacterial species are a special case because of their unusual cell wall that is decorated with unique glycoconjugates including lipoarabinomannan, lipomannan, PG-linked arabinogalactan, phosphatidylinositol mannosides, and mannoglycoproteins (Brennan and Nikaido, 1995; Jankute et al., 2015).

Glycan structures on the surfaces of both bacteria and their host cells are involved in many highly specific binding events that underlie bacterial pathogenesis (Poole et al., 2018). For example, glycans on the host cell surface can be recognized by bacterial lectins that function as adhesins to mediate attachment and invasion and to determine host cell and tissue tropism (Mahdavi et al., 2002; Spaulding et al., 2017). Host cell-surface glycans are also exploited by bacterial invaders as a carbon source (Johnston et al., 2007) and a receptor for bacterial toxins (Byres et al., 2008; Deng et al., 2014). Bacterial cell-surface glycans similarly promote adhesion to host cells through binding to host lectins (Colmenares et al., 2002; Tailleux et al., 2003). In addition to their roles in virulence, bacterial surface glycans are well known to help pathogens evade the host immune system, for example, by impairing the innate immune response through inactivation of the alternative complement pathway (Uria et al., 2008) or mimicking glycan structures present on host cells (Cress et al., 2014). These latter examples serve to highlight the impact of glycans on the immune system and their roles in pathogen recognition, modulation of innate immune responses, and control of immune cell homeostasis and inflammation.

Simply put, glycans and their glycan-binding partners play a role in nearly every aspect of immunology, a topic that is more thoroughly reviewed elsewhere (Avci and Kasper, 2010; Rabinovich et al., 2012; van Kooyk and Rabinovich, 2008). One representative example is the glycan-binding protein DC-SIGN, a C-type lectin receptor (CLR) found on the surface of dendritic cells (DCs) that recognizes a variety of mannose- and fucose-containing glycans — including glycoproteins that decorate the exterior of enveloped viruses such as human immunodeficiency virus (HIV), and signals into the cell for activation (van Kooyk and Geijtenbeek, 2003). Another example is CD22, a member of the sialic acid-bindingimmunoglobulin-like lectin (siglec) family that forms part of the B cell receptor (BCR) complex and assists with antigen uptake and B cell activation (O'Reilly et al., 2011). These and numerous other glycan-mediated interactions play crucial roles in shaping and directing the immune response and can also be exploited for vaccine purposes as we discuss below.

Given their important role in mediating interactions with the immune system, the surface carbohydrates of bacteria (Fig. 1) as well as other pathogens such as protozoa, helminths, viruses, and fungi have garnered attention as both vaccine antigens and adjuvants. In the context of vaccine antigens, surface carbohydrates of many pathogens contain epitopes that stimulate the adaptive arm of the immune response by activating B cells in healthy adults to produce protective antibodies against cell wall structures of the corresponding pathogen (Avci and Kasper, 2010; Rabinovich et al., 2012; van Kooyk and Rabinovich, 2008). This principle has been extensively exploited in various unconjugated and conjugated CPS- and LPS-based vaccine formulations (Ada and Isaacs, 2003; Astronomo and Burton, 2010; Jones, 2005; Weyant et al., 2018), some of which are now well established with more than four decades of clinical usage against *Haemophilus influenzae* type b (Hib), *Neisseria meningitidis*, *Salmonella enterica* serovar Typhi, and *Streptococcus pneumoniae*. In addition to bacterial pathogens, many tumor-associated carbohydrate antigens (TACAs) are being increasingly exploited as potential anti-cancer vaccines (Astronomo and Burton, 2010; Heimburg-Molinaro et al., 2011). In the context of vaccine adjuvants, some pathogen-associated carbohydrates are potent stimulators of the fast-responding innate immune system, which can be leveraged to enhance adaptive immune responses to co-administered antigens. One notable example is monophosphoryl lipid A (MPL), a Toll-like receptor 4 (TLR4) agonist that is derived from the natural LPS of *Salmonella minnesota* and the first non-alum vaccine adjuvant to achieve widespread clinical and market acceptance (O'Hagan et al., 2020; Pifferi et al., 2021). Alongside MPL and the saponin natural product QS-21 that is also part of several licensed vaccines, a variety of other naturally derived and chemically synthesized carbohydrate structures are in various stages of preclinical and clinical development. In addition to stimulating innate responses, carbohydrates can also enhance antigen immunogenicity by more efficiently delivering their linked antigens to specific immune cells in secondary lymphoid organs such as lymph nodes and spleens (Correia-Pinto et al., 2013; Liu et al., 2008). Finally, in the context of vaccine immunomodulators, carbohydrates can dampen immune adaptive and innate immune responses by shielding certain epitopes so as to refocus the immune response to specific, conserved regions on antigenic proteins (Hariharan and Kane, 2020) or by inducing tolerance to a co-.

administered antigen to suppress an ongoing immune response (Anderluh et al., 2021).

In this review, we highlight past and current efforts to harness the intrinsic properties of carbohydrates as vaccine components. The first part of this review focuses on the development of carbohydrates as vaccine antigens that activate adaptive immunity against the carbohydrate moiety and the factors that potentiate induction of affinity maturation and class switching of antibodies, or immunologic memory, which is a prerequisite for a successful vaccination. The second part of this review discusses the development of carbohydrates as vaccine adjuvants to increase the immunogenicity of co-administered antigens. Here, we consider two distinct ways that carbohydrates can enhance antigen-specific immune responses: (1) as ligands that activate innate immune cells; and (2) as delivery systems that promote specific immune cell or tissue tropism. Finally, we discuss how carbohydrates can also function as immunosuppressive agents that dampen undesired humoral immune responses to entire protein antigens or specific antigenic epitopes. Collectively, the reviewed works reveal the plurality of immunobiological functions that can be mediated by carbohydrates through their interactions with the adaptive and innate arms of the immune response, and how this knowledge is guiding the design and engineering of a new generation of highly efficacious glycan-containing vaccines against infectious pathogens, malignant cells, and complex immune disorders.

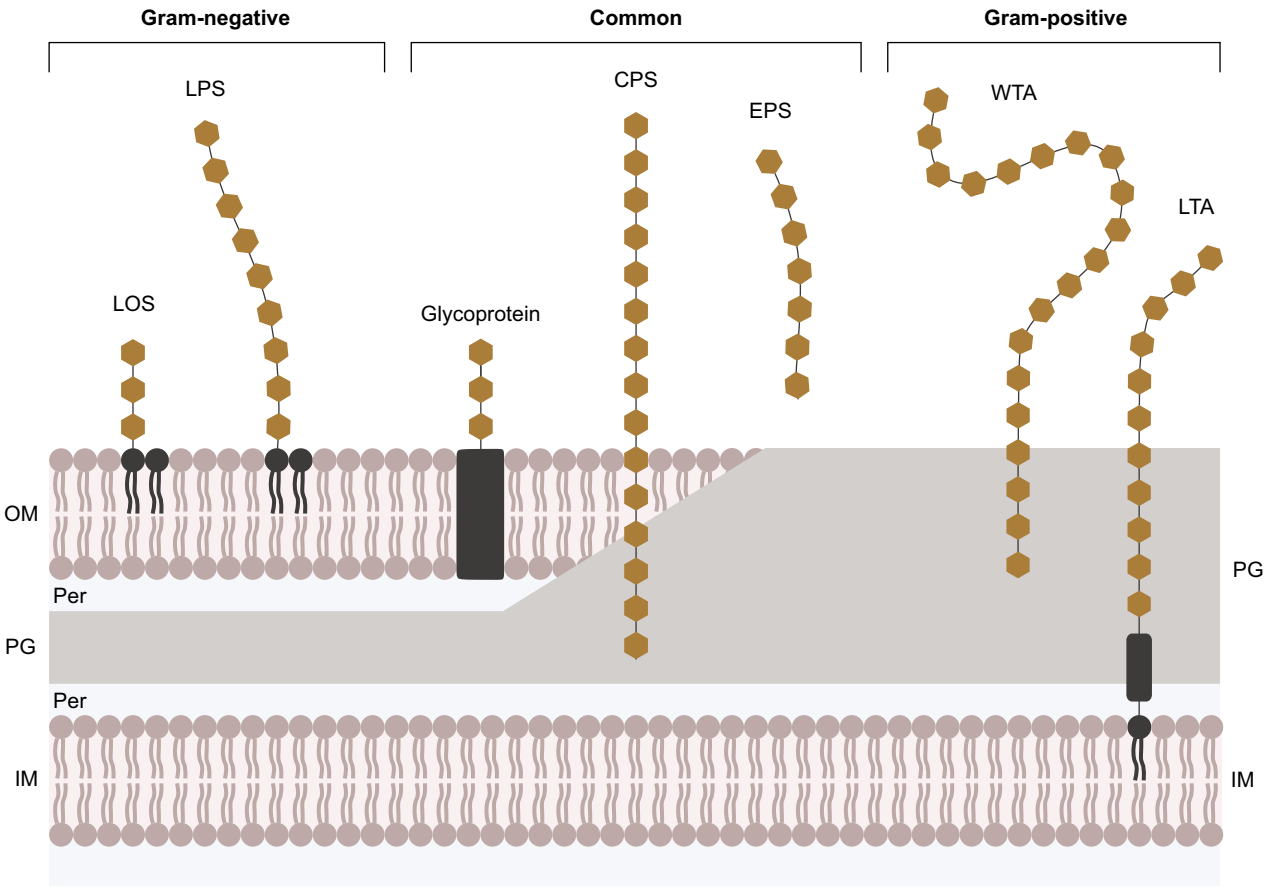

Fig. 1. Bacterial cell surface polysaccharides. Carbohydrate motifs on the surface of bacteria accessible to cells of the immune system are useful targets for raising a protective immune response. Some, including CPS and glycoproteins, are ubiquitous and present on many bacterial species. Other glycan structures are found primarily in gram-negative bacteria, such as LOS and LPS, or gram-positive bacteria, such as wall teichoic acid (WTA) and lipoteichoic acid (LTA). Some glycans, including lipoarabinomannan and arabinogalactan, are unique to mycobacteria, a subclass of gram-positive bacteria. Peptidoglycan (PG) is present on the surface of gram-positive but not gram-negative bacteria, where it is instead found in the periplasmic space (Per) between the inner membrane (IM) and outer membrane (OM).

2. Carbohydrates as vaccine antigens

2.1. Unconjugated polysaccharide vaccines and the requirement for T cell help

The adaptive immune response to carbohydrate antigens dates back to the 1920s and 30s with the discovery that serotype-specific CPS antigens from *S. pneumoniae* are immunoreactive (Francis and Tillett, 1930; Heidelberger and Avery, 1924) and elicit neutralizing antibodies that are protective against pneumococcal infection (Finland and Sutliff, 1932). Shortly thereafter, in 1945, the first CPS-based vaccine was reported in which a single subcutaneous immunization with polysaccharides corresponding to four different *S. pneumoniae* serotypes prevented pneumococcal pneumonia (Mac and Hodges, 1945). Several decades later, a 14-valent CPS-based vaccine comprising unconjugated CPS isolated from 14 different *S. pneumoniae* serotypes was commercially launched. The current incarnation, Pneumovax 23 includes 23 out of ~90 known serotypes and protects healthy adults against ~90% of infections caused by these pathogens (Daniels et al., 2016; Robbins et al., 1983). CPS vaccines involving unconjugated polysaccharides that target *N. meningitidis* serogroups A, C, Y and W-135 (Menomune-A/C/Y/W-135) and *S. enterica* serovar Typhi (Typhim Vi) have also been licensed.

The advent of these unconjugated CPS vaccines marked the establishment of carbohydrates as compelling, viable targets of a directed immune response. However, a major disadvantage of unconjugated polysaccharides is the lack of protective and memory responses, particularly in high-risk groups including neonates and children under two years of age, elderly, and immunocompromised people (Ada and Isaacs, 2003; Astronomo and Burton, 2010; Jones, 2005; Laferriere, 2011; Weyant et al., 2018). Pure carbohydrates including CPS, LPS, PG, and other glycans found on the bacterial surface are potent stimulators of the fast-responding innate immune system. They contain pathogen-associated molecular patterns (PAMPs) recognized by pattern recognition receptors (PRRs) including CLRs, TLRs, and Nod-like receptors (NLRs) found on the surface of DCs, macrophages, and other innate immune cells. Activation of PRRs by PAMP binding results in cytokine production that promotes inflammation and recruits effector cells. Despite their effective activation of the innate immune system, most polysaccharides are T cell-independent (TI) antigens that develop adaptive immune responses characterized by a lack of glycan-specific high-affinity antibodies as well as limited memory responses (Avci and Kasper, 2010; Rabinovich et al., 2012; van Kooyk and Rabinovich, 2008). The long, repetitive motifs present in bacterial polysaccharides crosslink BCRs on the surface of glycan-specific B cells to elicit the production of predominately low-affinity and short-lived immunoglobulin M (IgM) (Snapper and Mond, 1996) (Fig. 2a). Conversely, T cell-dependent (TD) antigens such as proteins elicit additional activation signals that facilitate class-switching and affinity-maturation processes, resulting in more high-affinity and long-lasting immunoglobulin G (IgG) antibodies in all age groups. These signals are provided by helper T cells that recognize antigen-derived peptides presented on major histocompatibility complex class II (MHCII) molecules (Yuseff et al., 2013).

While polysaccharides are generally classified as TI antigens, a

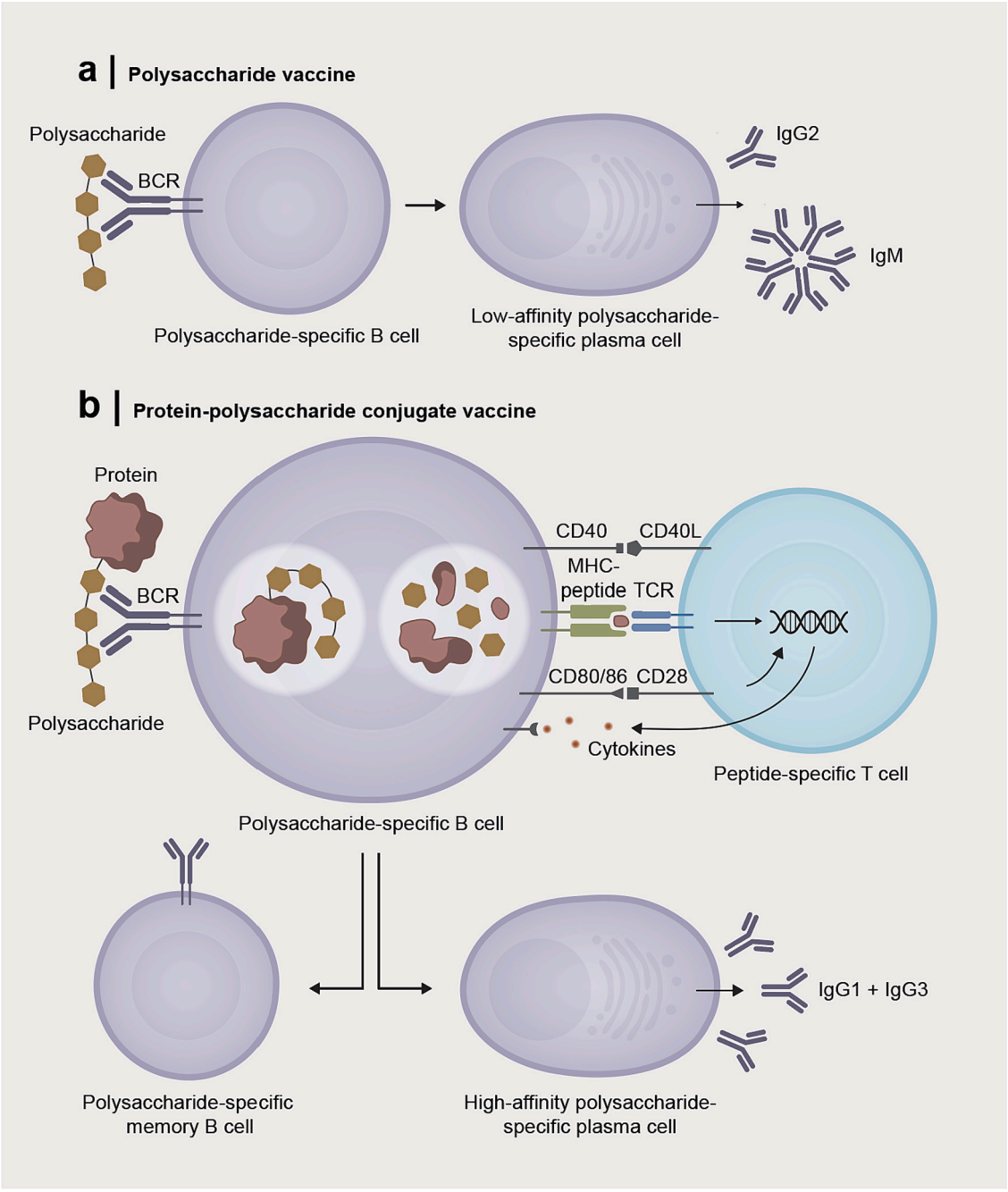

Fig. 2. Humoral immune response to carbohydrates. Antigen recognition and processing by carbohydrate-specific B cells is important in determining the nature of the corresponding immune response. Unconjugated polysaccharides can bind to multiple B cell receptors to elicit a TI response characterized by production of IgM with some class-switched IgGs (IgG2b and IgG3 in mice) as well as limited memory responses. Glycans conjugated to other biomolecules or that meet specific structural requirements may be processed intracellularly and bind to surface proteins on B cells for display and recognition by cognate T cells. Zwitterionic polysaccharides, peptides, and glycopeptides can be loaded onto MHC II for recognition by epitope-specific T cell receptors on T helper cells. Lipids or glycolipids, including analogues of α-galactosylceramide, are loaded onto CD1d and recognized by semi-invariant TCRs on invariant natural killer T cells. Co-stimulation between surface-displayed molecules including CD40 and CD40L on B cells and T cells, respectively, facilitates the release of cytokines that activate the B cell. TD responses are characterized by high-affinity, class-switched antibodies, and memory cell production.

notable exception are zwitterionic polysaccharides (ZPS) that form the capsules of some bacteria including *Bacteroides fragilis*, *Staphylococcus aureus*, and *S. pneumoniae* type 1. It has been shown that ZPS, which contain both positively and negatively charged carbohydrate residues in each repeating unit, are processed by antigen-presenting cells (APCs) and loaded onto MHCII, leading to activation of cognate T cells in a process that is indistinguishable from conventional protein antigens (Cobb et al., 2004). Along similar lines, the cell wall component TA from gram-positive bacteria has a zwitterionic state and activates T cells in an MHCII-dependent manner (Weidenmaier et al., 2010).

2.2. Conjugate vaccines

To overcome the poor immunogenicity of unconjugated polysaccharides in the most vulnerable populations, glycoconjugate vaccines in which the polysaccharide antigen is covalently attached to an immunogenic carrier protein were developed. Although it was known as early as 1931 that glycan conjugation to a carrier protein enhanced the immunogenicity of carbohydrates (Avery and Goebel, 1931), the enthusiasm for vaccination was dampened in the ensuing years due to the advent of chemotherapeutics and antibiotics. As a result, the first conjugate vaccines were not approved until the late 1980s (Jones, 2005). Since that time, numerous conjugate vaccines targeting the CPS of Hib, *N. meningitidis* and *S. pneumoniae* have been licensed and have dramatically decreased disease rates in the countries where they have been introduced (Grijalva et al., 2007; Halperin et al., 2012; Vella and Pace, 2015). Importantly, conjugate vaccines have proven to be among the safest and most effective vaccines developed over the last 40 years and have proven successful in populations at high risk for developing disease.

Numerous studies have demonstrated that conjugates elicit more desirable immune responses including protective, class-switched IgG antibodies and long-lasting immunological memory that are often absent in polysaccharide-only vaccines (Fig. 2b). Conjugate vaccines elicit a TD immune response through availability of T cell epitopes derived from the protein carrier (Rappuoli, 2018; Rappuoli et al., 2019). These short peptides, which are derived from intracellular processing of the carrier and binding to MHCII molecules on the APC surface, allow for the activation of corresponding T cells. B cells specific for the glycan antigen associate with these activated T cells and form an immune synapse before undergoing downstream processes such as antibody class-switching that underpins high-affinity antibody production. It was recently demonstrated that conjugate-derived glycopeptide epitopes can also be presented to T cells (Avci et al., 2011). At least in some cases, these epitopes can elicit more potent immune responses than peptide epitopes alone.

Most commercial glycoconjugate vaccines are based on polysaccharide antigens that are isolated from cultures of pathogenic bacteria and then conjugated to a separately produced carrier protein (Jones, 2005; Weyant et al., 2018). This process involves harvesting polysaccharides from the target pathogen after which the oligosaccharide of interest is purified, chemically activated, and finally chemically conjugated to a carrier protein such as tetanus toxoid (TT) from *Clostridium tetani*, diptheria toxoid (DT) from *Corynebacterium diphtheriae*, or CRM197, a DT mutant with a single amino acid substitution that ameliorates toxicity (Giannini et al., 1984). While effective, there are several challenges associated with traditional conjugate vaccines. Most notable among them is the complex, multistep process required for the isolation, chemical activation, and chemical conjugation of bacterial polysaccharides to a separately produced and purified carrier protein. This process is time-consuming, costly, and low yielding due to considerable losses that occur at each step. For example, only about 50% or less of the activated polysaccharide typically becomes conjugated (Frasch, 2009). Moreover, the final products are often ill-defined as a result of the heterogeneous mixture of glycans, random nature of chemical coupling, and activation chemistry, which can significantly reduce the size and change the physical structure of the polysaccharide, with loss of important epitopes that may have undesired effects on the immune response (Costantino et al., 2011). And because polysaccharide isolation requires large-scale cultivation of potentially hazardous bacteria, conjugate vaccine manufacturing is accompanied by biosafety regulations, centralized production, and high costs. Finally, the reliance on natural sources of polysaccharide antigens limits disease targets to those involving known carbohydrates that are well expressed and relatively easy to purify. Consequently, alternative approaches to conjugate vaccine synthesis have been explored.

One alternative involves chemical synthesis of the carbohydrate antigen moieties, which offers the potential for pure and homogeneous vaccines with a better safety profile while permitting the large-scale synthesis of complex oligosaccharides. A notable example is Quimi-Hib®, a commercially available Hib vaccine consisting of a synthetic capsular polysaccharide antigen of Hib conjugated to a known carrier protein that was found to compare favorably to licensed products when evaluated in clinical trials in Cuba (Verez-Bencomo et al., 2004). Chemical synthesis has been demonstrated for several other clinically important bacterial carbohydrate antigens including the repeat unit of PSII polysaccharide from *Clostridium difficile* (Adamo et al., 2012) the CPS of Group B *Streptococcus* (GBS), also known as *Streptococcus agalactiae* (Oldrini et al., 2020), and the conserved core LPS tetrasaccharide Hep_2Kdo_2 present on the surface of many bacterial pathogens (Kong et al., 2016). In addition to bacterial polysaccharides, chemical synthesis has also enabled the creation of synthetic tumor-associated carbohydrate antigens (TACAs) such as mucin-related Tn, STn, and T antigens, the gangliosides GM2, GD2, and GD3, and the glycosphingolipid globo H that target these aberrant glycan structures for cancer immunotherapy (Julien et al., 2009; Krug et al., 2004; Pifferi et al., 2020; Slovin et al., 1999; Wilson and Danishefsky, 2013). Beyond these semi-synthetic conjugates, synthesis schemes have also been described for fully synthetic immunogens consisting of a chemically synthesized carbohydrate antigen recognized by B cells connected to a chemically synthesized lipid or peptide carrier recognized by T cells. For example, a fully synthetic GM2-MPL conjugate involving the carbohydrate portion of human GM2 linked to MPL resulted in a potent, self-adjuvanting vaccine candidate (Zhou et al., 2017). Likewise, a fully synthetic, homogeneous glycopeptide immunogen bearing high-mannose *N*-glycans that mimics the glycan-polypeptide epitope within the native third variable loop (V3) of HIV-1 envelope (Env) glycoprotein was prepared by chemical synthesis and found to induce V3-glycan-directed antibodies (Alam et al., 2017).

Another alternative that is gaining traction involves cell-based conjugate biosynthesis using metabolically engineered *Escherichia coli* for one-step biosynthesis of an unlimited and renewable supply of conjugate vaccines (Kay et al., 2019) (Fig. 3a). This method, sometimes referred to as protein-glycan coupling technology (PGCT), leverages engineered protein glycosylation in non-pathogenic *E. coli* strains that are capable of enzymatically conjugating recombinantly produced CPS or O-antigen polysaccharide (O-PS) molecules to one or more engineered acceptor sites in co-expressed carrier proteins by an oligosaccharyltransferase (OST) such as the PglB enzyme from *Campylobacter jejuni* (Feldman et al., 2005). PGCT-based vaccines have been shown to elicit robust polysaccharide-specific immune responses against a range of bacterial pathogens including *Burkholderia pseudomallei*, *Francisella tularensis*, *Shigella* sp., and *S. aureus*, among others (Cuccui et al., 2013; Garcia-Quintanilla et al., 2014; Ihssen et al., 2010; Wacker et al., 2014), with a few currently under clinical investigation (Hatz et al., 2015; Huttner et al., 2017; Riddle et al., 2016). It should be noted that the expansion of PGCT to an even broader range of pathogens has been enabled by the identification of novel coupling enzymes and the optimization of existing ones (Duke et al., 2021; Harding et al., 2019; Ihssen et al., 2015), and numerous improvements to recombinant glycan expression, carrier protein design, and the bacterial host strain (Dow et al., 2020).

Building on these cell-based efforts, biosynthesis of conjugates has been accomplished using cell-free protein synthesis (CFPS) technology (Fig. 3b), which offers opportunities to both accelerate vaccine development and enable decentralized, cold chain-independent biomanufacturing by using cell lysates, rather than living cells, to synthesize proteins in vitro (Silverman et al., 2020). To date, CFPS systems have enabled on-demand and portable production of both aglycosylated DT vaccine proteins (Adiga et al., 2018; Pardee et al., 2016) as well as enzymatically glycosylated carrier proteins bearing bacterial polysaccharide antigens (Jaroentomeechai et al., 2018; Stark et al., 2021). In the latter instance, a method called iVAX (in vitro conjugate vaccine expression) was described that enables conjugate vaccine biosynthesis using cell-free extracts derived from glycosylation-

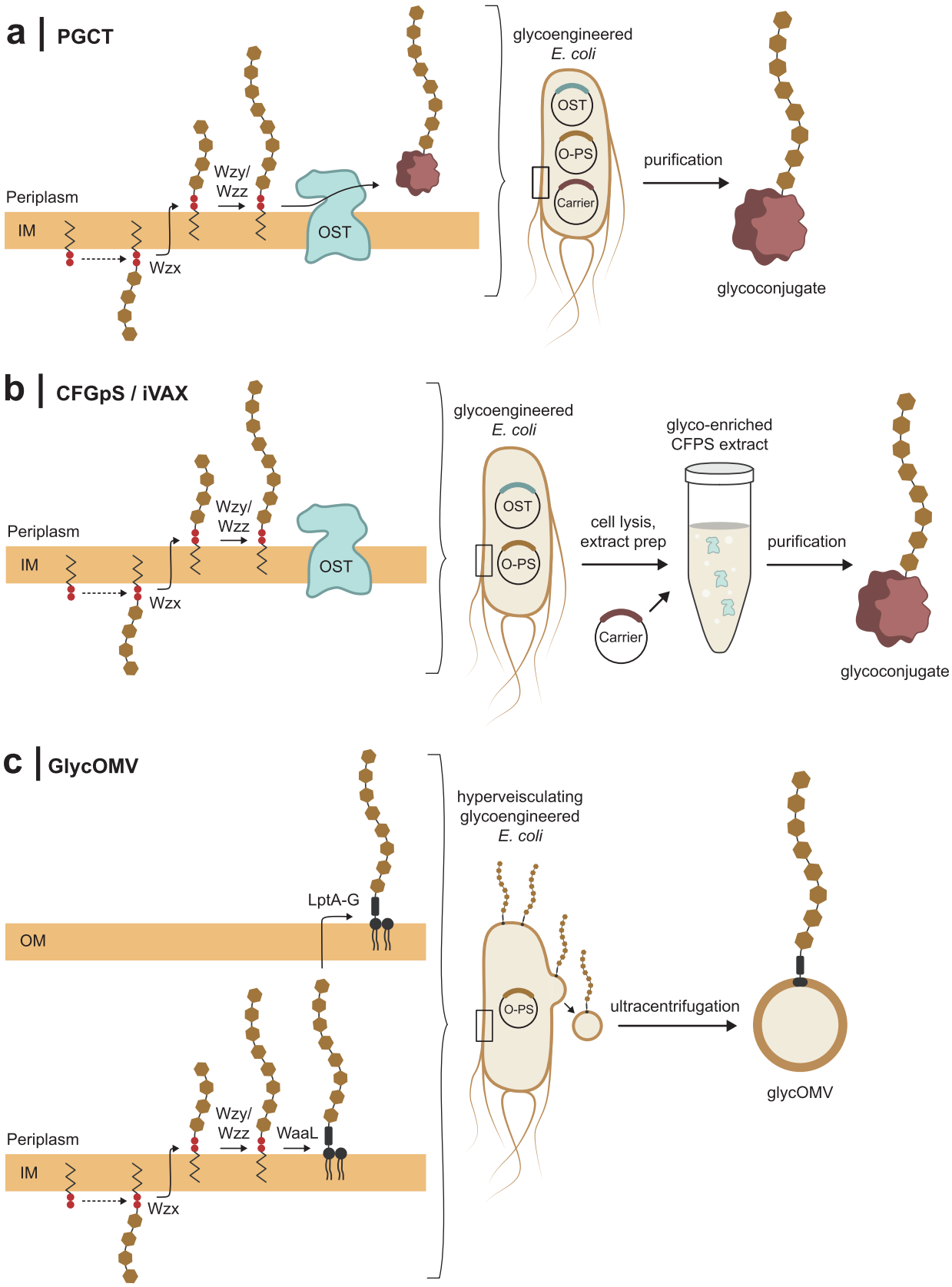

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Fig. 3. Glycoconjugate vaccine production using *E. coli* cells. (a) In PGCT, carbohydrate antigens are assembled on Und-PP in *E. coli*. The dashed line denotes a reaction step that is performed by several different enzymes, in this case, glycosyltransferases that are responsible for biosynthesis of a given O-PS structure. The glycan is flipped to the periplasm via the native *E. coli* flippase Wzx and may be polymerized via Wzy/Wzz. Heterologous expression of the OST from *C. jejuni*, namely PglB, is necessary to transfer the glycan to an acceptor sequence in a target carrier protein. (b) The iVAX platform involves the expression of an OST enzyme such as PglB and a pathogen-specific polysaccharide antigen, including CPS or O-PS, in nonpathogenic *E. coli* with detoxified lipid A, to produce low-endotoxin lysates comprising all machinery necessary for synthesis of conjugate vaccines. These reactions can be used to yield glycoconjugates containing licensed carrier proteins, and the platform provides a rapid means to develop and distribute conjugate vaccines against bacterial pathogens. (c) Carbohydrate antigens assembled on Und-PP can also be ligated to the core oligosaccharide of lipid A via WaaL, the native O-PS antigen ligase in *E. coli*. The lipid-linked antigen is then shuttled to the outer membrane where it subsequently becomes released in OMVs that are shed from the cell surface. The resulting glycOMVs can be isolated from the bacteria and used directly for vaccination.

competent *E. coli* strains that are enriched with a bacterial OST and pathogen-specific polysaccharide antigens, and subsequently primed with an immunostimulatory carrier protein plasmid (Stark et al., 2021). The modularity of the platform allows for facile switching of the carrier protein and bacterial polysaccharide antigen, facilitating the rapid manufacture of a range of vaccine candidates targeted against diverse bacterial pathogens. The effectiveness of the iVAX platform was demonstrated by the production of conjugate vaccines comprised of O-PS structures corresponding to *F. tularensis* and enterotoxigenic *E. coli* (ETEC) that stimulated strong O-PS-specific IgG antibody responses and protected mice against subsequent pathogen challenge (Stark et al., 2021; Warfel et al., 2023; Williams et al., 2023). Similar to cell-based conjugate vaccines, CFPS facilitates the production of conjugate vaccines with specific positioning of polysaccharides on carrier proteins by integrating non-canonical amino acids (Kapoor et al., 2022) or engineering multiple attachment sites for multivalent vaccine design (Fairman et al., 2021).

Because of the dramatic simplification of the cell-based and cell-free processes, bioconjugation strategies allow scalable production of large quantities of conjugates at a much more affordable cost, which is especially important for achieving sustained impact on global public health. Indeed, large-scale manufacturing processes for conjugates offer a highly competitive cost per dose as described for *E. coli*-based production of a biologic antiviral (Decker et al., 2020) and cell-free production of conjugate vaccine candidates (Stark et al., 2021). With further optimization of the reaction formulation, the cell-free system can produce a vaccine dose for ~$0.50 assuming a dose size of 10 μg and the lyophilized reactions are stable for up to four weeks of storage at room temperature, 37 °C and 50 °C (Warfel et al., 2023). The ability to freeze-dry cell-free system components for distribution and storage at ambient temperature and reconstitute by just adding water presents the potential to extend on-site manufacturing and promote broader access to conjugate vaccines. Additional advantages of cell-free systems for conjugate production include linear scaling from 1 nL to 100 L for accelerated process development, rapid customization and reconfiguration for product switching, and circumvention of biosafety concerns associated with the use of living cells outside of a controlled laboratory setting. Collectively, the cell-based and cell-free platforms described above lay the foundation for future creation of a custom, multivalent vaccine with the potential for broad serotype coverage and increased access through adoption of simplified, low-cost biomanufacturing platforms.

A final variation on the original covalent conjugate theme makes use of outer membrane vesicles (OMVs), which are spherical nanoparticles released from the outer membranes of all gram-negative bacteria. OMVs are composed of proteins, lipids, and glycans, including CPS and LPS, derived primarily from the bacterial outer membrane and periplasm (Kulp and Kuehn, 2010). OMVs have garnered significant attention as vaccines because they are nonreplicating, immunogenic mimics of the originating bacteria that stimulate both innate and adaptive immunity and possess intrinsic adjuvant properties (Kaparakis-Liaskos and Ferrero, 2015). To date, native OMVs isolated directly from *N. meningitidis* have been successfully incorporated into a multicomponent vaccine formulation called Bexsero® that has been licensed for use in humans (Gorringe and Pajón, 2012). Outer membrane protein complexes from *N. meningitidis* have also been used as carriers in licensed conjugate vaccines (Scaria et al., 2019). The vaccine potential of OMVs has been further expanded using genetic engineering techniques to load OMVs with heterologously expressed antigens, resulting in structures that stimulate immune responses that are strong, antigen-specific, and protective (Chen et al., 2010; Rappazzo et al., 2016). While most efforts to date have focused on foreign peptide and protein antigens, recent work has demonstrated that the exterior of OMVs can be decorated with CPS or O-PS antigens (Fig. 3c). For example, expression of heterologous polysaccharide biosynthesis pathways in hypervesiculating *E. coli* cells yielded glycosylated OMVs (glycOMVs) displaying pathogen-mimetic polysaccharides such as *N. meningitidis* polysialic acid and *F. tularensis* O-PS as well as tumor-mimetic glycans such as the T antigen (Chen et al., 2016; Valentine et al., 2016). The resulting glycOMVs elicited high titers of polysaccharide-specific antibodies and, in the case of *F. tularensis*, protected mice against lethal pathogen challenge. Along similar lines, broadly protective glycOMVs engineered by surface expression of the conserved polysaccharide antigen, poly-*N*-acetyl-D-glucosamine (PNAG), and its deacetylated counterpart, dPNAG, in hypervesiculating *E. coli* cells (Stevenson et al., 2018). Mice immunized with PNAG/dPNAG-containing glycOMVs produced serum antibodies that mediated efficient in vitro killing of two distinct PNAG-positive bacterial species, namely *F. tularensis* subsp. *Holarctica* and *S. aureus*, and developed protective immunity against these unrelated pathogens. It is noteworthy that the PNAG/dPNAG-containing glycOMVs performed as well or better than a TT-based conjugate in terms of immunogenicity and protection, highlighting the potential of glycOMVs for vaccine applications.

In addition to their strong immunogenicity and self-adjuventicity, the reactogenicity of glycOMVs can also be engineered by remodeling the lipid A structure. For example, deletion of the acyltransferase-encoding *lpxM* gene in the host strain genome combined with co-expression of *F. tularensis* phosphatase LpxE resulted in a glycOMV-producing host strain that generated a structurally homogeneous mimic of MPL. The glycOMVs produced by this strain exhibited dramatically reduced endotoxicity relative to those containing native *E. coli* lipid A molecules without any adverse effects on their immunogenicity (Chen et al., 2016; Stevenson et al., 2018), although suitability of the detoxified glycOMVs for human use remains to be assessed.

Alongside covalent conjugates, several innovative strategies have also emerged for non-covalently assembling polysaccharide antigens with effective carrier molecules. One example is the multiple antigen-presenting system (MAPS) that was developed to replicate the immunological and antigenic strengths of whole cell vaccines using an acellular, macromolecular complex to stimulate multipronged immune responses (Zhang et al., 2013). In the MAPS vaccine platform, the covalent polysaccharide-protein linkage found in conventional glycoconjugates is replaced by an affinity-based association between biotinylated polysaccharides and biotin-binding fusion proteins. The resulting MAPS complexes, loaded with CPS from *S. pneumoniae* serotype 14 (CPS14), elicited protective B- and T-cell-mediated immunity against pneumococcal disease. Recently, a 24-valent pneumococcal vaccine, AFX3772, was developed using the MAPS system and shown to be immunogenic and safe in adults (Chichili et al., 2020). A conceptually similar technology known as protein capsular matrix vaccines (PCMVs) involves entrapment of a polysaccharide antigen in a cross-linked

protein matrix with little to no direct covalent attachment between the capsular antigen and protein (Thanawastien et al., 2015). PCMVs composed of CPS14 or *Bacillus anthracis* poly-gamma-D-glutamic acid (PGA) antigen elicited anticapsular antibody responses that were comparable to that elicited by commercial conjugate vaccines, with the CPS14-based PCMV inducing isotype antibody switching and immunological memory. Similar to multivalent MAPS preparations, multiple distinct polysaccharides can be entrapped in the same PCMV simultaneously to create multivalent formulations. Collectively, these technologies substantiate the notion that covalent conjugation to carrier proteins is not an absolute requirement for transforming TI polysaccharide antigens into more effective TD immunogens.

3. Carbohydrates as adjuvants

3.1. Subunit vaccines and the need for adjuvants and immune stimulators

Subunit vaccines built from fragments of pathogens are desirable because of their ability to elicit immune responses that specifically target rationally chosen antigens, while also providing excellent safety profiles compared to their whole-pathogen counterparts (Pollard and Bijker, 2021). However, the precision and safety of subunit vaccines often comes at the cost of inferior immunogenicity and protective efficacy as they are not as easily recognized by immune cells compared to whole-pathogen vaccines. Other liabilities of subunit vaccines include low permeability and oral absorption due to their high molecular weight and hydrophilic character. In addition, subunit vaccines are susceptible

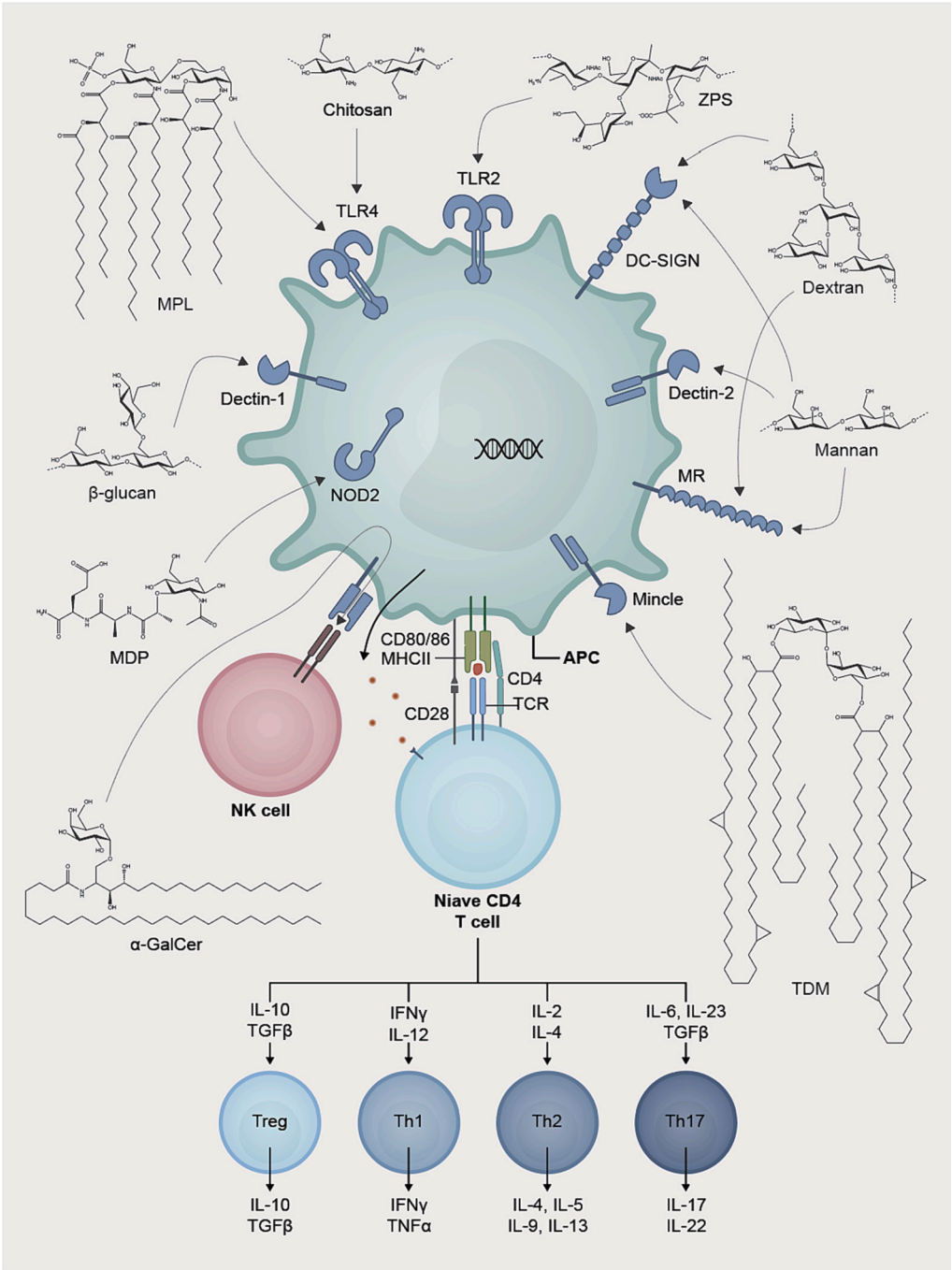

Fig. 4. Representative carbohydrate-based vaccine adjuvants that activate innate receptors. Carbohydrates can interact with diverse receptors that initiate innate and adaptive downstream pathways. Monophosphoryl lipid A (MPL) is a TLR4 agonist that triggers the release of proinflammatory cytokines as well as co-stimulatory molecules required for the adaptive immune response. Chitosan also interacts with the TLR4 receptor complex, activating inflammasome formation and the DNA-sensing cGAS-STING pathway. Dextran binds to mannose-binding receptors and DC-SIGN, leading to NFκB-mediated secretion of proinflammatory cytokines. Mannans bind various receptors, including mannose-binding receptors, DC-SIGN, and Dectin-2, which also trigger proinflammatory cytokine production. The glycolipid α-GalCer is presented to and activates NKT cells through CD1d presentation. Trehalose 6,6′-dimycolate (TDM) binds macrophage-inducible C-type lectin (Mincle). β-glucans bind Dectin-1 on APCs as well as other PRRs such as CR3 on myeloid immune cells. Zwitterionic polysaccharides (ZPS) activate TLR2-dependent pathways, leading to cytokine and co-stimulatory molecule production. Lastly, muramyl dipeptide (MDP) interacts with NOD2, a cytoplasmic receptor, which leads to NFκB-mediated proinflammatory cytokine release. Adapted from (Stefanetti et al., 2021).

to enzymatic degradation and are thus characterized by short half-lives in vivo. Consequently, subunit vaccines typically require the assistance of adjuvants and/or delivery systems. However, while many licensed subunit vaccines have adjuvants in their formulations, only a handful have adequate potency and sufficiently low toxicity for use in humans, highlighting the need for new adjuvants.

Due to their ability to mimic the key features of pathogens, carbohydrates hold great potential as natural and relatively safe vaccine adjuvants and immune stimulators that enhance antigen-specific immune responses and engage specific immune pathways tailored towards the elimination of specific pathogens or cancers (O'Hagan et al., 2020; Pifferi et al., 2021). Carbohydrates elicit immune responses following recognition by specific glycan-binding receptors such as CLRs, NLRs, TLRs, and siglecs found on the surface of various immune cells (Avci and Kasper, 2010; Rabinovich et al., 2012; van Kooyk and Rabinovich, 2008) (Fig. 4). The binding event activates various intracellular signaling pathways that initiate a cascade of innate and adaptive immune responses. Moreover, carbohydrates can also function as delivery vehicles that transport their antigen cargo to secondary lymphoid organs including lymph nodes, spleen, tonsils, and certain tissue in various mucous membrane layers in the body. Of the limited number of vaccine adjuvants that have been approved in human vaccines, two are natural carbohydrate structures: bacterially derived MPL and plant derived QS-21 (Kensil et al., 1991; Qureshi et al., 1982). Carbohydrate-based adjuvants are typically derived from natural carbohydrates or else prepared by chemical synthesis. As discussed elsewhere (Stefanetti et al., 2021), this section examines several of the most promising carbohydrate-based adjuvants with a focus on the inherent properties (e.g., immunomodulation activity, biocompatibility, biodegradability, tolerability, safety, etc.) that make them such a versatile and useful class of macromolecules for development as adjuvants.

3.2. Bacterial glycan-based adjuvants

Bacterial LPS molecules contain a highly immunogenic lipid A moiety that can bind TLR4 on APCs and elicit innate immune responses (Raetz and Whitfield, 2002). While lipid A is endotoxic in humans, endotoxicity-attenuated variants can act as potent adjuvants. One such variant, MPL, is a lipid A variant derived from *S. minnesota* R595 (Qureshi et al., 1982) that activates TLR4 (Evans et al., 2003) and triggers the biosynthesis of diverse mediators of inflammation such as TNF-α IFN-γ, IL-6, and IL1-β as well as co-stimulatory molecules required for the adaptive immune response (Komai-Koma et al., 2021; Okemoto et al., 2006). The induction of inflammation by MPL augments APC recruitment and antigen uptake (De Becker et al., 2000). The ability of MPL to modulate innate immune responses has resulted in its use, either alone or in combination with a second adjuvant such as alum or the saponin QS-21 (see below), in licensed vaccine products against hepatitis B, human papillomavirus, malaria, *N. meningitidis*, pollen allergies, and shingles (herpes zoster) (O'Hagan et al., 2020). Despite these successes, a major challenge with bacterially derived lipid A molecules including MPL is the high heterogeneity of the preparations as well as possible contaminations from other inflammatory components, which directly impacts the adjuvanticity and safety profile. To circumvent this issue, synthetic chemistry approaches have been employed to obtain well-defined semi-synthetic or fully synthetic lipid A molecules with tailored fatty acid acylation and phosphorylation patterns that display greatly reduced toxicity while maintaining most of the immunostimulatory activity of the parental LPS or MPL (D'Alonzo et al., 2016; Gaekwad et al., 2010; Zhang et al., 2007). It is also worth mentioning that in addition to its use as an unconjugated component in anti-infection vaccines, conjugation of MPL to the antigen of interest has been investigated as a self-adjuvanting anti-cancer vaccine candidate (Zhou et al., 2015). Both natural and synthetic lipid A molecules possess unusual immunoreactivity, with the latter class holding promise in the search for new vaccine adjuvant candidates.

As mentioned above, some bacteria such as *B. fragilis, S. pneumoniae, and S. aureus* produce cell surface ZPS with characteristic patterns of positively and negatively charged carbohydrate residues within each repeat unit. It has been shown that both natural and chemically derived ZPS can activate APCs via a TLR2-dependent mechanism, induce production of co-stimulatory molecules and cytokines, and induce $CD4^+$ T cell activation in vitro (Gallorini et al., 2007). Hence, in addition to their ability to activate adaptive immune responses (Cobb et al., 2004), ZPS have additional immunostimulatory properties that motivate their use as adjuvants. Indeed, when ZPS were co-administered as adjuvants together with an unconjugated antigen such as TT, increased antigen-specific antibody titers were observed in mice (Gallorini et al., 2009), revealing the inherent adjuvant activity of ZPS. Moreover, conjugation of CRM_{197} with ZPS generated by chemical introduction of positive charges into anionic polysaccharides from GBS resulted in ZPS-CRM_{197} conjugates that induced higher antibody and T-cell responses to the PS and carrier, respectively, compared to control conjugates made with the native polysaccharide form (Gallorini et al., 2009). This increased immunogenicity was attributed to the ability of ZPS to activate DCs in a TLR2-dependent manner. Other semi-synthetic approaches have been explored including the construction of entirely carbohydrate-based conjugate vaccines incorporating *B. fragilis* ZPS molecules, namely PS A1 and PS B, with various TACAs including Tn, STn and T antigen (De Silva et al., 2009; Shi et al., 2016; Trabbic et al., 2016). These entirely carbohydrate-based immunogens elicited strong TACA-specific antibody and T cell responses in mice that in some cases were stronger than the responses elicited by a traditional protein carrier-TACA conjugate. It should also be noted that total synthesis of ZPS repeating units has been reported for a number of different bacterial structures (Eradi et al., 2018; Keith and Townsend, 2019; Visansirikul et al., 2015), yielding useful building blocks for constructing ZPS-based adjuvants that may overcome the low yields that are commonly achieved with natural and semi-synthetic ZPS. However, to date, these fully synthetic ZPS have yet to be thoroughly evaluated for adjuvanticity.

Heat-inactivated mycobacterial components in oil emulsions comprise Freund's Complete Adjuvant (FCA), which is widely used as an adjuvant in experimental vaccines. However, while FCA elicits strong humoral and cellular responses in animals and humans, toxicity hampers its use in a clinical setting. A search for well-defined components that constitute the minimal immunostimulating component in FCA led to the discovery of muramyl dipeptide (MDP), a glycosylated dipeptide consisting of *N*-acetyl muramic acid linked to L-alanine-D-isoglutamine (Ellouz et al., 1974). The ability of MDP and its derivatives to elicit adjuvant activity has since been extensively demonstrated (Ogawa et al., 2011). These compounds achieve adjuvanticity through binding to NOD2, a cytoplasmic receptor belonging to the human innate immune system, which leads to activation of NF-κB and MAPK and subsequent release of proinflammatory cytokines (Coulombe et al., 2009). Another component of mycobacterial cell wall, trehalose-6,6′-dimycolate (TDM), also has potent immunostimulatory activity. TDM, which consists of a trehalose disaccharide esterified with two long-chain mycolic acid chains, activates immune cells to confer adjuvant activity through interactions with macrophage-inducible C-type lectin (Mincle) and macrophage C-type lectin (MCL) (Furukawa et al., 2013), The high reactogenicity of TDM renders it unsuitable for use in humans, leading to the development of synthetic TDM analogs that are similarly potent but less toxic, such as trehalose-6,6′-dibehenate (TDB). The TDB analog illustrates how advances in synthetic chemistry in the context of immunobiology contribute to developing structurally defined, potent adjuvant molecules.

The α-glucan dextran is a branched microbial polysaccharide made of α-1,6-glucan with α-1,3-branches that binds to mannose-binding receptors and DC-SIGN on macrophages and DCs, leading to NF-κB-mediated secretion of proinflammatory cytokines (Chieppa et al., 2003; Li et al., 2018). Owing to its immunostimulatory properties, dextran has been investigated as a vaccine adjuvant. For example, acetalated

dextran (Ac-DEX) has been demonstrated to increase MHC-I/MHC-II presentation of a model protein antigen, ovalbumin (OVA), in vitro (Broaders et al., 2009) and enhance humoral and cellular responses to an M2e-based influenza vaccine in vivo (Chen et al., 2018a). To date, however, no dextran derivatives have achieved success as human adjuvants.

3.3. Eukaryotic glycan-based adjuvants

Saponins are plant-derived glycosides with a triterpenoid aglycone core attached to one or more branched or linear polysaccharides. Saponins extracted from the bark of the Chilean *Quillaja saponaria* tree were the earliest to be evaluated for their adjuvanticity. One extract, QS-21, is a mixture of isomeric saponins QS-21-apiose and QS-21-xylose and the most widely used saponin due to its potent immunostimulatory properties, excellent tolerability in mammals, and abundant availability (Kensil, 1996). While the exact mechanism of QS-21 adjuvanticity remains unclear, it is postulated that it binds to CLRs on APCs through its carbohydrate residues and becomes internalized, after which it stimulates a T_H1-biased immune response with production of high antibody titers as well as antigen-specific cytotoxic T lymphocytes (Kensil, 1996). QS-21 is formulated as part of the AS01 adjuvant system, together with MPL, that is used in licensed vaccines against malaria and shingles (O'Hagan et al., 2020). As with some of the bacterially-derived adjuvants above, QS-21 has several inherent liabilities including heterogeneity and low yields from natural sources and dose-limiting toxicity that have limited its clinical use as a stand-alone adjuvant. To address these limitations, semi-synthetic and synthetic analogs of QS-21 have been reported, providing access to potent, structurally defined adjuvants with the potential to tailor the immunostimulation profiles (i.e., balanced versus biased T_H1/T_H2 response) (Wang et al., 2005; Wang et al., 2019). Interestingly, in addition to its demonstrated potential in anti-infection vaccines, QS-21 has also been investigated as an adjuvant in an experimental Alzheimer's vaccine involving Aβ1–7 peptide conjugate, with the addition of QS-21 being required to elicit sustained release of high, sustained of anti-Aβ antibody titers (Arai et al., 2015).

α-Galactosylceramide (α-GalCer) is a marine sponge-derived glycolipid that consists of a galactose monosaccharide attached to a phytosphingosine with an amide-linked saturated acyl chain. The acyl chain of α-GalCer binds to a non-polymorphic, MHC-I-like receptor CD1d on DCs and subsequently becomes presented to a special subset of T cells known as invariant natural killer T cells (iNKT cells) that bridge the innate and adaptive immune systems (Kawano et al., 1997) by triggering the production of both T_H1-type (IFNγ) and T_H2-type (IL-4) cytokines (Kawano et al., 1997). Consequently, αGalCer and its synthetic analog, KRN7000, have been investigated as vaccine adjuvants and found to improve both humoral and cellular immunity when co-administered with various vaccines (Yates et al., 2018). Since synthetic α-GalCer analogs can preferentially elicit a T_H1- or T_H2-dominant immune response based on their structure (Goff et al., 2004; Li et al., 2010; Trappeniers et al., 2008), they can potentially be used as highly modular adjuvants in vaccines to elicit pathogen-tailored immune responses.

β-glucans are linear or branched β-1,3-glucose polysaccharides that are part of fungal cell walls and are recognized by the CLR Dectin-1 on APCs as well as other PRRs such as CR3 on myeloid immune cells. Receptor binding promotes cytokine production and B- and T-cell activation, leading to enhanced humoral and cellular immune responses (Brown and Gordon, 2003). As vaccine adjuvants, β-glucans stimulate innate and adaptive immunity in different ways depending on their source, structure, and formulation. For instance, particulate β-glucan activates DCs and macrophages via the dectin-1 pathway whereas soluble β-glucan primes innate neutrophils for adjuvant activity via complement and CR3-dependent pathways (Qi et al., 2011). In clinical studies, the soluble form of β-glucan was co-administered with a bivalent tumor vaccine consisting of two gangliosides GD2 and GD3 conjugated to the carrier protein, KLH, and found to greatly augment the anti-GD2 and anti-GD2 antibody titers (Kushner et al., 2014). The adjuvant activity of β-glucans has also been exploited by conjugation of the polysaccharide to protein (CRM_{197}) or peptide (MUC1) antigens, yielding conjugates that enhanced antigen-specific antibody responses (Donadei et al., 2015; Wang et al., 2018).

Chitosans are linear polysaccharides consisting of β-1,4-linked glucosamine and *N*-acetylglucosamine residues that are found in the exoskeletons of crustaceans and cell walls of fungi. They bind to TLR4 (Zhang et al., 2014), activating inflammasome formation and the DNA-sensing cGAS-STING pathway that elicits secretion of type-I interferons and enhances phagocytosis in macrophages and NK cells (Carroll et al., 2016). As a vaccine adjuvant, chitosan potentiates humoral and cellular responses to co-administered antigens through a depot effect and by activating macrophages and NK cells via phagocytosis to produce inflammatory cytokines (Amidi et al., 2010; Zaharoff et al., 2007). To date, numerous preclinical studies of vaccine adjuvants based on chitosans have been carried out, assessing their ability to increase immunogenicity and potentiate both humoral and cell-mediated immune responses to various vaccine antigens, (Bal et al., 2010; Ghendon et al., 2008; Zaharoff et al., 2007).

Fructans are plant-derived polymers of fructose with terminal glucose residues. δ-inulin, a β-D-[2 → 1]polyfructofuranosyl-α-D-glucose polysaccharide, is a clinically licensed adjuvant named Advax™, which is used in numerous antibacterial and antiviral vaccines (Petroski, 2017). Advax™ uses a microcrystalline form of δ-inulin that elicits a balanced T_H1/T_H2 response and enhances the production of various cytokines and interferons that aid $CD4^+$ and $CD8^+$ T cell proliferation (Saade et al., 2013). The precise mechanism of immune activation remains elusive, but highly desired for further improvements in the potency, safety, and applicability of δ-inulin as vaccine adjuvant.

Mannans are β-1,4-mannose polysaccharides that constitute the cell walls of plants and fungi and potentiate immune responses by enhancing antigen presentation and TLR4-dependent DC maturation (Sheng et al., 2006). Mannans bind a wide variety of receptors expressed by APCs including TLRs, DC-SIGN, mannan-binding lectin (MBL), and CLRs of the mannose receptor family such as Dectin-2 (Cambi et al., 2008; Choteau et al., 2016; Jouault et al., 2003; Saijo et al., 2010). Stimulation of these receptors on APCs triggers intracellular pathways that produce proinflammatory cytokines such as INF-γ and IL-12 that promote antigen uptake and presentation by APCs (Borriello et al., 2022; Mathiesen et al., 2019) as well as increased T_H1/T_H2 responses (Apostolopoulos et al., 1995). As vaccine adjuvants, mannans have been conjugated to protein and peptide antigens such as synthetic MUC1 glycopeptides, eliciting high antigen-specific antibody titers but weak cytotoxic T cell responses (Karanikas et al., 1997) and indicating that mannan-dependent adjuvanticity polarizes the immune response towards antibody production over cell-mediated immunity. It is also worth noting that conjugation of mannans to the vaccine antigen is not necessary for its adjuvanticity as unconjugated mixtures of mannans with inactivated H1N1 virus elicited higher titers of serum IgG and respiratory-tract IgA than inactivated H1N1 conjugated to mannan, or H1N1 alone (Proudfoot et al., 2015).

4. Carbohydrates as delivery systems

4.1. Carbohydrate-based delivery for improved systemic and mucosal immunity

In addition to their use as adjuvants that are promptly recognized by PRRs of the innate immune system such as CLRs, TLRs, and NLRs, carbohydrates can also protect against antigen degradation, exhibit mucoadhesive properties, and are usually biodegradable and safe for use in humans (Bashiri et al., 2020; Mosaiab et al., 2019). In light of these properties, carbohydrates and their derivatives are among the most promising vaccine delivery platforms currently under development. It should be noted, however, that the distinction between carbohydrates as

targeting molecules and carbohydrates as adjuvants is ambiguous because these roles are interconnected and overlapping. Indeed, glycan-based adjuvants can function as targeting molecules by engaging specific elements of the immune system, including innate and adaptive components, to generate a more robust and enduring immune response. Therefore, to avoid confusion related to the potential dual nature of carbohydrates as adjuvants and targeting molecules, in this section, we define carbohydrate-based delivery systems as those that can enhance systemic and mucosal immunity against co-administered antigens via routes other than (or in addition to) PRR engagement.

Carbohydrates can enable prolonged circulation and controlled release of adjuvants and/or antigens from delivery particles. The degradation rate of carbohydrate-based nano- and micro-particles significantly impacts delivery and subsequent immune cell engagement. For example, Ac-DEX has been explored as a tunable delivery vehicle (Bachelder et al., 2008; Broaders et al., 2009; Chen et al., 2018b; Suarez et al., 2013). The degradation rate of Ac-DEX can be modulated by varying the ratio of cyclic acetal which hydrolyzes more slowly and acyclic acetal which hydrolyzes faster (Broaders et al., 2009). By tuning the degradation rate of Ac-DEX, a notable enhancement in MHC-I presentation efficacy was achieved relative to PLGA or Ac-DEX with suboptimal degradation properties. Furthermore, antigens enclosed within Ac-DEX particles that degrade rapidly were presented through a TAP-independent pathway, while those within slowly degrading Ac-DEX were not. In addition, the delivery particle size is an important determinant of vaccine effectiveness (Bachmann and Jennings, 2010; Joshi et al., 2013).

Carbohydrates are particularly effective in delivering vaccines to mucosal and epithelial tissues. Antigens delivered through mucosal routes (e.g., nasal and oral) are taken up by microfold cells (M cells) in mucosa-associated lymphoid tissue (MALT) of the gut, nasal passage, and bronchi, among others (Mabbott et al., 2013). To overcome barriers like pH, enzymatic degradation, and rapid clearance, there is significant focus on using pH-sensitive and mucoadhesive carbohydrate-based carriers to enhance the immune response of vaccines delivered mucosally (Xing et al., 2019). The hydroxyl groups present on carbohydrate molecules allow for non-covalent bioadhesion to biological tissues, including mucosal and epithelial membranes, which can improve drug targeting (Mosaiab et al., 2019) and prolong interactions between associated antigens and immune cells. In addition, certain carbohydrates can disrupt tight junctions and directly transfer antigens through epithelial barriers (Dodane et al., 1999). Below we provide key examples of important carbohydrates for mucosal or dermal vaccine delivery.

Chitosan is a cationic polymer that readily forms nano- and micro-particles that enable encapsulation of vaccine antigens and protection from degradation prior to uptake by APCs (Koppolu and Zaharoff, 2013). The resulting chitosan-based nano- and micro-particles have been broadly investigated as a vaccine delivery platform (Dmour and Islam, 2022; Mourya and Inamdar, 2009). Chitosan has been shown to enhance the transmucosal uptake, and thus increase the bioavailability of small molecules, peptides, and proteins (Amidi et al., 2010; Illum, 2012; Mills et al., 2003). ChiSys®, a chitosan-based delivery platform, has shown promise in preclinical and clinical investigations for intranasally administered vaccine antigens (Atmar et al., 2011; El-Kamary et al., 2010). For instance, a norovirus virus-like particle (VLP) vaccine containing ChiSys® as a mucoadhesive agent was delivered intranasally to human test subjects and significantly reduced the frequencies of Norwalk virus gastroenteritis (Atmar et al., 2011). Extensive studies have been conducted on derivatives of chitosan as well. The solubility of chitosan at physiological pH may result in pre-systemic drug metabolism in the presence of proteolytic enzymes (Xing et al., 2019), rendering it suboptimal for particles intended to circulate within the body that are not soluble or stable below neutral pH. To overcome this limitation, various chemical modifications have been implemented, such as quaternization, alkylation, and phosphorylation (Dmour and Islam, 2022). Trimethyl chitosan (TMC) is one of the most commonly used chitosan derivatives and has been employed for the delivery of DNA (Thanou et al., 2002) and protein antigens from influenza A (Amidi et al., 2007; Dabaghian et al., 2018; Liu et al., 2015), hepatitis B (Tafaghodi et al., 2012), SARS-CoV-2 (Jearanaiwitayakul et al., 2021), and more. For example, intranasal administration of influenza A subunit H3N2-loaded TMC nanoparticles in mice yielded significantly stronger IgG and hemagglutination-inhibiting immune responses than co-administration with free antigen or TMC solution containing soluble antigen (Amidi et al., 2007).

Cellulose-based systems have been explored for the mucosal delivery of DNA (Song et al., 2014), killed virus (Waldman et al., 1986), whole cell (Lin et al., 1991), and subunit (Maharaj et al., 1984) vaccines. Certain cellulose derivatives are valuable for oral drug delivery because they can resist dissolution under strongly acidic conditions, such as the stomach, but readily dissolve in mildly acidic or neutral environments such as the intestine (Roxin et al., 1998). For instance, a vaccine prepared by loading freeze-dried *Mycoplasma hyopneumoniae* into cellulose acetate phthalate (CAP) microspheres resisted pH-related inactivation and protected orally immunized pigs from mycoplasmal pneumonia (Lin et al., 1991; Weng et al., 1992).

Hyaluronic acid (HA) has been explored for the transdermal delivery of vaccines. HA can rapidly absorb through the epidermis and into the dermis of the skin (Brown et al., 1999). In light of this ability, HA nanocapsule formulations decorated with OVA as a model antigen have been evaluated in ex vivo studies using a pig skin model and found to retain OVA in higher quantities in the skin compared to the antigen in PBS (Bussio et al., 2019). In addition, the vaccine activated complement via C3/C3b cleavage in studies using human plasma from healthy donors. Interestingly, high molecular weight HA is generally considered inert to immune cells owing to its ubiquitous presence in the extracellular matrix (Gariboldi et al., 2008). However, at sites of inflammation, HA is broken down into fragments of low molecular weight that can activate immune signaling cascades (Gariboldi et al., 2008; Termeer et al., 2002). Therefore, HA may have the potential to serve as an inert or self-adjuvanting vaccine delivery vehicle.

5. Carbohydrates as dampeners of immune responses

5.1. The role of glycans as immune shields and the rise of immunofocusing

From the standpoint of vaccine development, carbohydrates are perhaps best known for their use as vaccine antigens and adjuvants. However, they also hold potential in other aspects of vaccine design owing to their ability to exert a dampening effect on innate and adaptive immune responses. To this end, it is now firmly established that many human viruses including, for example, HIV-1, influenza, coronaviruses, Ebola, and Lassa exploit host-cell machinery to glycosylate their own proteins during replication (Watanabe et al., 2019). The envelope proteins associated with these viruses such as envelope glycoprotein (Env) of HIV-1, hemagglutinin (HA) of influenza A, and spike (S) glycoprotein of SARS-CoV-2 are common targets for glycosylation. Moreover, *N*-linked glycosylation sites occur regularly at antibody-sensitive locations that are often highly conserved regions of the envelope protein surface (Doores et al., 2010; Stewart-Jones et al., 2016; Wang et al., 2009; Watanabe et al., 2020). Because *N*-linked glycans are flexible, bulky structures, more than 20 times the size of an amino acid side-chain and are typically nonimmunogenic or weakly immunogenic due to strong negative selection of autoreactive B and T cells, the glycan units effectively shield the underlying amino acids from antibody recognition. Indeed, modification of antibody-sensitive sites in envelope proteins with “self” glycans provides a protective camouflage or shield that helps the virus to evade immune recognition (Kobayashi and Suzuki, 2012; Skehel et al., 1984; Walls et al., 2016; Wei et al., 2003). By masking surfaces that might otherwise be sites of effective antibody-mediated neutralization, the glycan shield contributes to the persistence of viral infection and enables the virus to sidestep conventional B cell-based vaccine approaches (Fig. 5a). It is worth noting that similar glycan-

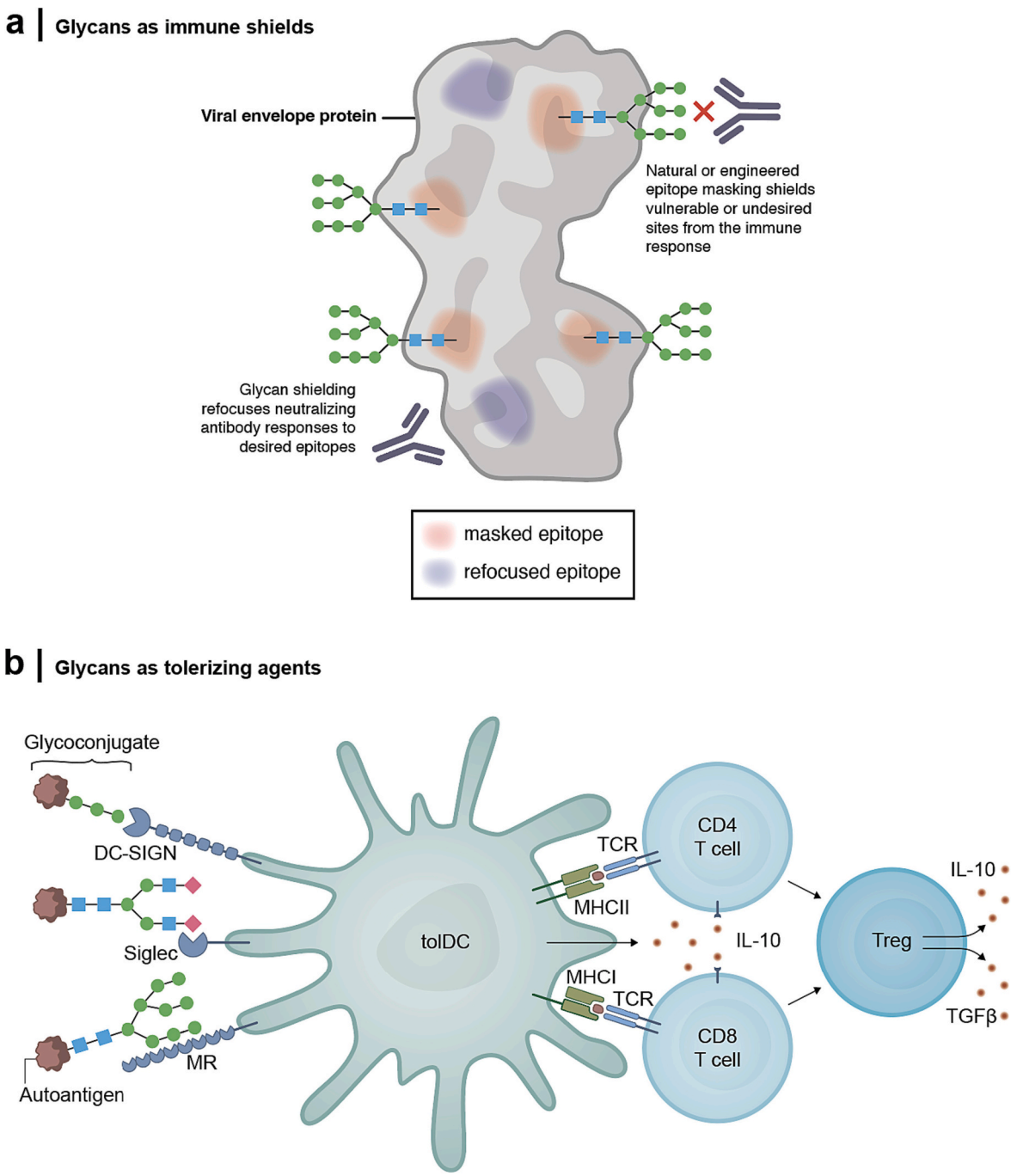

Fig. 5. Glycan-mediated dampening of immune responses. (a) For many viral envelope proteins, glycans shield vulnerable protein epitopes, such as the receptor binding site, from neutralizing mAbs, while non-vulnerable protein epitopes lack selective pressure for glycan shielding. From a vaccine design perspective, glycosylation sites can be engineered into immunogens such that B-cell responses are steered away from undesired antigenic sites and are refocused to desired epitopes, a strategy known as immunofocusing. (b) In the case of tolerogenic immunotherapies, DCs endocytose glycoconjugates after the recognition of the self-glycan motif by DC-SIGN, siglecs, or CLRs such as the mannose receptor (MR). This leads to the differentiation of the DC into a tolerogenic DC (tolDC) and the presentation of autoantigen in complex with MHC-II or MHC-I. Antigen recognition by CD4+ and CD8+ T cells in the presence of secreted IL-10 leads to their differentiation into Tregs. Upon recognition of the autoantigen in the periphery, Tregs prevent the onset of autoimmunity by secreting IL-10 and TGF-β.

mediated immune evasion tactics are employed by pathogenic bacteria that exploit "self" glycan mimics in their CPS or LPS as a means of avoiding immune attack (Cress et al., 2014).

Inspired by these natural pathogenic camouflaging strategies, many groups have explored rational glycan shielding whereby additional *N*-glycans are introduced at undesired antigenic sites of a protein-based immunogen such that B-cell responses are refocused to the desired epitopes without compromising the immunogen's overall folded.

structure (Hariharan and Kane, 2020). This method of glycan-mediated "immunofocusing" is most commonly used to steer the immune response to specific, highly conserved regions on the antigen that are known to be recognized by broadly neutralizing antibodies (Fig. 5a). For example, in HIV-1 the major target for neutralizing antibodies (NAbs) is the Env complex formed by gp120/gp41, with the principal neutralizing determinant located in the third hypervariable domain (V3) in the gp120 subunit. While antibodies directed against non-V3 epitopes

are known to neutralize a more diverse array of HIV-1 isolates, they are generated at lower titer and occur later during natural infection (Kang et al., 1991). It is believed that the V3 domain serves as an immunodominant decoy epitope that keeps the immune response somewhat fixed and limited by suppressing maturation of the later pool of broadly neutralizing, low-titer Abs. This suppression of a primary immune response to other antigenic determinants is thought to be one of the reasons for limited vaccine efficacy. To overcome this immunodominant epitope, glycan masking of the V3 domain has been performed through site-specific addition of *N*-linked glycans to a gp120 immunogen, resulting in antigenic dampening without loss of gp120/CD4 binding or virus neutralization (Garrity et al., 1997). Importantly, selective dampening of the V3 epitope steered the dominant NAb response away from V3 to an epitope in the first variable domain (V1) and to conserved epitopes in the C1 and C5 domains of gp120. Collectively, these seminal results demonstrated how site-directed epitope masking with *N*-glycans can induce demonstrable shifts in the immune response, resulting in second-order neutralizing responses that could lead to more broadly protective vaccines. Since this early demonstration of glycan-mediated immunofocusing, numerous studies have reported similar epitope masking design strategies that have yielded glycoengineered vaccine immunogens against HIV-1 (Duan et al., 2018; Pantophlet et al., 2003; Selvarajah et al., 2005; Selvarajah et al., 2008), influenza virus (Chen et al., 2019; Chen et al., 2021; Eggink et al., 2014; Lin et al., 2012; Lin et al., 2014), coronaviruses (Du et al., 2016; Lin et al., 2021), and dengue and Zika viruses (Lin et al., 2019). It is also worth noting that a conceptually similar approach to glycan-based immunofocusing involves removal of structurally non-essential glycans on viral surface glycoproteins. This strategy involves glycodeletion of viral envelope proteins, either by enzymatic trimming to remove already installed *N*-glycans or site-directed mutagenesis to remove encoded glycosylation sequences, and has proven useful against influenza and coronavirus (Chen et al., 2014; Huang et al., 2022; Wang et al., 2009; Wu et al., 2022).

While immunofocusing has proven to be a generalizable strategy for selective epitope dampening of immunogens based on viral envelope proteins, few studies have investigated whether site-directed introduction of one or more additional *N*-glycans in non-viral protein antigens can also elicit selective epitope dampening. In one notable example, it appears that glycan shielding can indeed be extended to other proteins (Sliepen et al., 2015). For example, the widely used GCN4-based isoleucine zipper (IZ) protein trimerization domain is known to be highly immunogenic, inducing potent anti-IZ antibody responses in animals. However, following site-directed installation of four *N*-linked glycans in the IZ domain that were predicted to shield the underlying protein surface, antibody responses against the IZ domain were strongly reduced whereas responses to its unmodified fusion partner were unaffected. Hence, immunosilencing of immunogenic multimerization domains with glycans appears to be a relevant deimmunization strategy for designing less immunogenic vaccine antigen components that could also be extended to therapeutic proteins.

5.2. *Tolerizing the immune system with mono- and multivalent glycoconjugates*

A final opportunity for harnessing glycosylation of antigens that we consider here is the use of glycoconjugates for eliciting tolerogenic immune responses (Anderluh et al., 2021). Because glycosylation is tissue- and individual-specific, the composition of glycans at the cell surface provides a unique signature for the immune system to discriminate between self and non-self (Varki, 2006). As such, innate and adaptive immune cells are equipped with glycan-binding proteins that can either augment the immune response upon recognition of pathogenic or foreign glycans or suppress the immune response in the presence of self-glycans (Alves et al., 2022). Thus, changes in the glycosylation signature of cells and tissues during the disruption of homeostasis, such as long periods of inflammation and cancer, can result in a loss of tolerance and the concomitant induction of unwanted humoral immune responses to protein antigens. These responses are characterized by the presence of autoreactive T and B cells and are responsible for numerous medical conditions in the areas of autoimmunity, allergies, transplantation, and biotherapeutics (Gould and Sutton, 2008; Kwun et al., 2012; Naparstek and Plotz, 1993; Singh, 2011).

To address these immune tolerance-related disorders, a desirable approach is to silence or delete the antigen-reactive lymphocytes in a manner that preserves protective immunity (Miller et al., 2007). Natural mechanisms for suppressing B cell activation through their BCR inhibitory coreceptors such as the siglec CD22 (Siglec-2), which recognizes sialic acid-containing glycans, represent attractive targets for directly tolerizing B cells in an antigen-specific manner. However, since the majority of CD22 is not associated with the BCR, its association with the BCR must be enforced to exploit its inhibitory effect on B cell activation. Along these lines, two separate studies used synthetic polymers co-displaying the TI antigen nitrophenol together with glycan ligands of CD22 and showed that physically tethering CD22 and the BCR suppressed B cell activation (Courtney et al., 2009; Duong et al., 2010). This concept was extended to TD type antigens whereby liposomal nanoparticles that co-displayed both a protein antigen and CD22 glycoligands, known as STALs (SIGLEC-engaging tolerance-inducing antigenic liposomes), were observed to induce tolerance to proteins via deletion of the antigen-reactive B cells by apoptosis (Macauley et al., 2013). STALs with hen egg lysozyme (HEL), OVA, myelin oligodendrocyte glycoprotein (MOG), and human factor VIII (FVIII) were tolerogenic, resulting in significantly lower antibody responses following a challenge with the corresponding antigen. In the case of FVIII, the ability of STALs to prevent an undesired antibody response was found to protect mice from bleeding in a tail-cut assay following administration of hFVIII.

In addition to antigen-reactive lymphocytes, DCs have also shown promise as targets for tolerogenic immunotherapies due to their ability to bridge the innate and adaptive immune systems (Fucikova et al., 2019). These strategies aim to tolerize DCs for the re-education of adaptive lymphocytes and the restoration of antigen-specific tolerance by delivering glycosylated autoantigens that bind to PRRs on the surface of DCs such as CLRs and siglecs. Among these, the mannose receptor selectively binds carbohydrate groups such as mannose and fucose on glycoproteins of yeast, bacteria, parasites, and certain viruses and can function as an endocytic receptor that promotes antigen presentation and activates anti-inflammatory responses (Geijtenbeek and Gringhuis, 2009). Mannose receptor-mediated endocytosis is a very efficient process allowing multiple rounds of antigen binding and endosomal delivery and can result in up to a 10,000-fold increased efficiency of presentation of mannosylated antigen compared to non-mannosylated antigen (van Bergen et al., 1999). This mechanism has been exploited by decorating antigens with mannose, resulting in glycoconjugates that induce a state of tolerance to experimental autoimmune encephalomyelitis (EAE). Specifically, it is known that the encephalitogenic peptide $PLP_{139–151}$ induces EAE in mice. However, mice immunized with a mannosylated conjugate of $PLP_{139–151}$ did not develop disease and did not show significant inflammation of the central nervous system (CNS) when evaluated shortly after the peak of disease activity (Luca et al., 2005). After re-immunization with $PLP_{139–151}$ in complete adjuvant, mice that had been immunized with mannosylated $PLP_{139–151}$ were largely tolerant to EAE, showed less T cell proliferation, and exhibited decreased peptide-specific IgG2a, suggesting that the mannosylated peptide induced active suppression or deletion of peptide antigen-specific T cells. The resolution of EAE has also been shown through a similar strategy involving sialylation of antigens. Specifically, chemical conjugation of MOG with either α2,3- or α2,6-linked sialyl-lactose resulted in sialylated glycoconjugates that were endocytosed by DCs through binding to Siglec-E, leading to differentiation of CD4+ T cells into regulatory T (Treg) cells and decreased effector CD4+ and CD8+ T cells (Perdicchio et al., 2016). Along similar lines, allergoids conjugated

to non-oxidized mannan have been developed as hypoallergenic vaccines targeting DCs that may provide a new strategy for inducing tolerance to allergens such as grass pollen (Sirvent et al., 2016). These allergen-mannan conjugates enhanced allergen uptake and promoted healthy T-cell responses to allergens, generating functional Tregs and inducing a shift to non-allergic reactions in a DC-SIGN-specific manner. Collectively, these studies demonstrate how carbohydrate-conjugated antigens can provide a novel way to induce antigen-specific immune tolerance that may lead to new immunotherapeutic strategies for autoimmune diseases and allergies.

While the results of using glycoconjugates in the modulation of autoimmune diseases and allergies are promising, further studies are needed to characterize the universality of this strategy, especially when targeting glycan receptors that can induce both pro- and anti-inflammatory responses. Moreover, long-term studies are needed to determine if the induction of tolerance protects the individual indefinitely and if the repetition of the same treatment upon relapse is equally effective. Similarly, it is important to determine both the effects that the route and location of immunization have in other myeloid cells that express the same glycan-binding receptors and the conditions in which immunization could worsen disease progression.

6. Conclusion

Many decades after the first successful campaign, vaccination remains the most cost-effective and efficacious of all potential approaches for infectious disease prevention. Nonetheless, there is an urgent need for continued advancements in vaccinology especially in light of predictions that drug-resistant bacteria may threaten up to 10 million lives per year by 2050 (O'Neill, 2014). In the ongoing search for better and safer vaccines, carbohydrates have attracted considerable attention because of their ubiquitous presence on the surface of all living cells, viruses, and parasites as well as the vital role they play in mediating interactions with both the innate and adaptive immune systems. Indeed, glycans and their glycan-binding partners play a role in almost every facet of immunology. The works reviewed here collectively display the range of immunobiological activities that can be facilitated by carbohydrates through their myriad relationships with the adaptive and innate segments of the immune response, and how this expanding understanding is informing the engineering and design of increasingly efficacious vaccines comprising glycan-based antigens, adjuvants, delivery systems, and immune dampeners. Greater knowledge of how carbohydrates interact with the innate and adaptive arms of the immune response, along with further advances in the field of synthetic glycobiology, will allow for additional improvements in the design of novel carbohydrate-containing vaccine formulations and immunomodulators capable of fighting and preventing both emerging and established diseases.

Declaration of Competing Interest

M.P.D. and M.C.J. have financial interests in Gauntlet, Inc. and Resilience, Inc. M.P.D. also has financial interests in Glycobia, Inc., MacImmune, Inc., UbiquiTX, Inc., and Versatope Therapeutics, Inc. M.P.D.'s and M.C.J. interests are reviewed and managed by Cornell University and Stanford University, respectively, in accordance with their conflict-of-interest policies. All other authors declare no competing interests.

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