Glycan-Based Cell Targeting To Modulate Immune Responses

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Abstract

Glycosylation is an integral post-translational modification present in more than half of all eukaryotic proteins. It affects key protein functions including folding, stability, and immunogenicity. Glycoengineering approaches, such as the use of bacterial *N*-glycosylation systems, or expression systems including yeasts, insect cells, and mammalian cells, have enabled access to defined and homogenous glycoproteins. Because glycan structures on proteins can be recognized by host lectin receptors, they may facilitate cell-specific targeting and immune modulation. Myeloid C-type lectin receptors (CLRs) expressed by antigen-presenting cells are attractive targets to shape immune responses. Multivalent glycan display on nanoparticles, liposomes or dendrimers has successfully enabled CLR targeting. This review discusses novel strategies to access defined glycan structures and highlights CLR targeting approaches for immune modulation.

Glycoimmunology – glycobiology meets immunology

Glycans play essential roles in various biological processes such as cell proliferation and differentiation, organism development, and the immune response. However, in contrast to peptides or nucleotides, glycans have been undervalued by scientists for a long time, mainly due to their complexity [1]. Glycans are not directly encoded in the genome; instead, their biosynthesis is dictated by metabolism, signal transduction, and cellular status [2]. In recent years, significant progress has been made in both the structural elucidation of complex glycans and the assembly of defined glycan structures and glycoproteins using bioengineering approaches and chemical synthesis. These advances have stimulated research in glycoimmunology including profiling glycan–lectin interactions in the immune system. This review highlights recent advances in obtaining defined glycan structures and glycoproteins and details how glycans can be used to target receptors in innate immunity, particularly C-type lectin receptors (**CLRs**, see Glossary). The main focus will be on the multivalent presentation of glycans on carrier systems such as nanoparticles, liposomes, or dendrimers and how these tools can be used for targeting immune cell subsets.

Novel glycoengineering approaches towards defined glycoproteins

Because glycans are often required for proper folding and biological activity of proteins, neither removing *N*-glycosylation sites nor attempting protein expression in prokaryotes such as *E. coli*, which lack a functional *N*-glycosylation machinery, is usually suitable for the production of therapeutic glycoproteins [3]. Hence, suitable expression systems for glycosylated proteins have been developed, including expression in bacteria with a functional glycosylation machinery; expression in eukaryotic cells such as yeasts, insect cells, protozoa, plants, mammalian cells; and cell-free expression systems (Figure 1). An initial breakthrough was the transfer of the *N*-linked glycosylation system from *Campylobacter jejuni* to *E. coli* [4]. Since then, major progress has been made in glycoprotein expression in **bacteria** [5]. The

transfer of four eukaryotic glycosyltransferases and the bacterial oligosaccharyltransferase PglB from *C. jejuni* into *E. coli* enabled the production of eukaryotic trimannosyl chitobiose glycans and their transfer to eukaryotic proteins [6]. Glycosylation machineries have successfully been transferred from other bacterial species to *E. coli* as well. A recent example is the functional transfer of the glycosylation system from the δ-proteobacterium *Actinobacillus pleuropneumoniae* to *E. coli* [7]. *A. pleuropneumoniae* encodes an unusual pathway for *N*-linked protein glycosylation which takes place in the cytoplasm. The process is catalyzed by a soluble *N*-glycosyltransferase that uses nucleotide-activated monosaccharides to glycosylate asparagine residues. This enzyme may prove useful for the production of glycoproteins that form inclusion bodies which can be then be purified and folded *in vitro*. Thus, this study provides the basis for a novel route for engineering *N*-glycoproteins in bacteria [7].

Many protein drugs are glycoproteins, including hormones, cytokines, monoclonal antibodies, antibody–drug conjugates, or glycoconjugate vaccines [8]. Therefore, it is crucial to produce proteins that display a uniform, human-like glycosylation. In human glycoproteins, complex, high-mannose, and hybrid *N*-glycans are present. In contrast, glycoproteins derived from **yeasts** are naturally hypermannosylated (Figure 2), making it necessary to eliminate the genes responsible for yeast-specific glycosylation and replace them with genes encoding for human glycoenzymes. A glycoengineered strain of the yeast *Pichia pastoris* could produce glycoproteins with fully complex terminally sialylated *N*-glycans [9]. Recent studies focused on engineering the O-linked glycosylation pathway, making it possible to produce sialylated O-linked glycans in yeast [10]. The discovery of a nucleocytoplasmic O-mannose glycoproteome in yeast opened up possibilities to manipulate nucleocytoplasmic O-mannose glycosylation for subsequent bioprocessing [11].

Glycoproteins from **insect cell lines** carry high mannose *N*-glycans as well as truncated, paucimannosidic *N*-glycans (Figure 2). In the baculovirus-insect cell expression system, this

limitation has been addressed by introducing mammalian genes encoding for protein *N*-glycosylation functions [12]. Recent advances in glycoengineering insect cell lines include installing inducible promoters for mammalian protein *N*-glycosylation [13] or applying the CRISPR-Cas technology to modify protein glycosylation pathways [14].

Mammalian cell lines are most frequently used to produce glycoprotein drugs because the resulting glycan profile is most similar to that of human proteins [15]. However, although mammalian cell lines produce glycoproteins with complex, human-like *N*-glycans, they may also display the immunogenic terminal galactose-α1,3-galactose (α-Gal) epitope and the non-human sialic acid *N*-glycolylneuraminic acid (Neu5Gc, Figure 2). A recent study reported a novel glycoengineering strategy, called GlycoDelete, to gain access to glycoproteins with homogeneous *N*-glycans. By shortening the Golgi *N*-glycosylation pathway in mammalian cells, the authors were able to produce glycoproteins carrying small, sialylated trisaccharide *N*-glycans with a markedly reduced *N*-glycan heterogeneity compared to glycoproteins derived from native mammalian cells [16]. While bioengineering methods for the expression of glycoproteins in bacteria are so far limited to the incorporation of a few glycan chains, the GlycoDelete approach may also be suitable for the expression of higher glycosylated proteins with defined glycosylation patterns.

Furthermore, bioprocess parameters may markedly impact glycosylation patterns of glycoproteins. For instance, the cell line [17], cell density, media composition [18], nutrient levels and supplements [19, 20], pH value, and transfection conditions are of major importance. Glucose deprivation was shown to impair antibody glycosylation and thus resulted in hypoglycosylated antibody glycoforms in Chinese Hamster Ovary CHO cells, the most frequently used mammalian cell line for glycoprotein production [21, 22]. The interactions among these parameters are complex, so attention must be paid to process development and optimization to produce glycoproteins with desired glycosylation profiles [23].

Finally, **chemoenzymatic and synthetic strategies** have helped to generate defined *N*-glycan structures and glycoproteins [24]. Recent examples include the synthesis of the glycosylated hormone erythropoietin [25], homogeneous human glycosyl-IFN-β [26], and glycopeptide antigens from the HIV gp120 glycoprotein [27]. Advances in site-selective protein glycosylation, namely the "tag-and-modify" approach, which involves the installation of a uniquely reactive chemical group into a protein (the "tag") and the subsequent selective modification of this group, have alleviated access to homogenous glycoproteins [28, 29]. Further progress in chemical and chemoenzymatic approaches for site-specific protein modification [30, 31] will pave the way towards well-defined, human-like glycoproteins.

Protein glycosylation impacts immunogenicity

It is well known that glycosylation has a major influence on the immunogenicity of proteins (Figure 3). Examples include the α-Gal epitope, which is not present on human *N*-glycans and may cause anaphylactic reactions, or the non-human sialic acid Neu5Gc, which induces antibody responses upon dietary absorption and may accumulate in human tissues [3]. Another prominent example is glycosylation of the antibody Fc domain, which affects recognition by Fcγ receptors, thus inducing signal pathways leading to either inflammation or immune modulation [32]. The immunomodulatory effect induced by antibody/Fcγ receptor interactions is exploited in the therapy of autoimmune disorders by administering intravenous immunoglobulins (IVIG) [33]. However, antibody/Fcγ receptor interactions may also induce robust anti-tumor responses. While short-term anti-tumor responses are caused by tumor cell killing via FcγRIII-mediated cytotoxicity (ADCC), Fc receptors are also involved in the induction of long-term cellular immune responses against tumors, as recently demonstrated in an elegant study using FcγR-humanized mice [34]. This study indicates that next-generation anti-tumor antibodies should be designed for binding to specific Fcγ receptors. Optimal Fcγ receptor engagement by antibodies is also important during infectious diseases. One study

144 compared the effector functions of different *N*-glycoforms of palivizumab, a humanized mAb
145 used for the immune prophylaxis against respiratory syncytial virus [35]. Enhanced Fcγ
146 receptor binding was associated with reduced viral lung titers, indicating the importance of
147 antibody Fc glycosylation for antiviral activity. In line with these results is another study that
148 compared glycoengineered antibodies with enhanced FcγRIIIa binding affinity with respect to
149 their effector functions in macrophages [36]. Under physiologic conditions, glycoengineered
150 antibodies exhibited increased FcγRIIIa binding and promoted enhanced macrophage
151 functions indicated by an increased antibody-dependent phagocytosis and cytotoxic activity.
152 While the impact of Fc glycosylation on immunogenicity is well known for IgG, recent data
153 indicate that IgE glycosylation is required during allergy. Genetic disruption of the *N*-
154 glycosylation site in the Fc domain of IgE or enzymatic removal of the oligomannose glycan
155 inhibited common features of allergic reactions such as mast cell degranulation accompanied
156 by the release of biologically active inflammatory mediators [37]. In conclusion,
157 glycosylation may markedly impact glycoprotein recognition by lectins of the immune
158 system. However, glycosylation may also modify the infectivity and transmission of
159 pathogens. While the recognition of pathogen-derived glycans on glycoproteins and other
160 glycoconjugates by lectins on immune cells may finally lead to a protective immune response
161 on the one hand, lectin/glycan interactions can also be exploited by pathogens for immune
162 evasion on the other hand. For instance, two recent studies on cell-culture based influenza
163 vaccines indicated that differential *N*-glycosylation of hemagglutinin, a major envelope
164 protein of the influenza virus, markedly impacted its immunogenicity and affected antibody
165 levels and hemagglutinin inhibition titers *in vivo* [38, 39]. In another recent study, the effect of
166 *N*-glycosylation of simian immunodeficiency virus (SIV) envelope proteins on viral spread
167 and immunogenicity was investigated [40]. Decoration of the envelope with high-mannose *N*-
168 glycans resulted in reduced viral infectivity and mucosal transmission, whereas viral capture
169 by immune cell lectins was enhanced. This study suggests that viral *N*-glycosylation can have

opposed effects on SIV infectivity and lectin recognition and further supports the importance of lectins in the recognition of glycan structures on pathogens.

Lectin receptors as targets for cell-specific delivery and immune modulation

The aforementioned examples highlight the potential of exploiting glycan-binding receptors as targets for immune modulation. In particular, three major classes of lectins in innate immunity are promising targets: (i) siglecs, which are cell surface receptors mainly expressed by immune cells which bind to glycans carrying sialic acid residues, thus regulating immune responses; (ii) galectins, a lectin family defined by their specificity for β-galactoside sugars; and (iii) CLRs, which often bind carbohydrates in a Ca^{2+}-dependent fashion. For instance, siglec targeting is a means to modulate immune responses. Indeed, cancer cells often exhibit an increased degree of sialylation to inhibit NK cell activation through recruitment of Siglec-7 [41]. Antigen targeting to Siglec-7 using a high-affinity ligand led to rapid endocytosis and robust T cell responses, suggesting the utility of siglec targeting for cell-specific antigen delivery [42]. Another example is the siglec Sialoadhesin (CD169) on macrophages, which was efficiently targeted using glycan ligand-decorated liposomes [43]. Siglecs may also be exploited to selectively suppress immune responses to autoantigens. In a recent study, targeting of Siglec-2 (CD22) using antigenic liposomes resulted in robust tolerance to protein antigens in mice by inducing B cell apoptosis in an antigen-specific manner [44]. Similar to siglecs, galectins are often involved in immune regulation, but have a wide variety of additional functions including cell–cell interactions or apoptosis. Several galectins have been implicated in tumorigenesis and metastasis and, consequently, galectins may hold potential for selective tumor targeting and as candidate biomarkers for tumor imaging [45, 46].

CLRs represent a large lectin superfamily including, among other classes, the collectins, selectins, and the myeloid CLRs (Figure 4, and Box 1). CLR targeting may be exploited to affect antigen-presenting cell (APC) functions and to shape immune responses. Indeed, CLR

196 targeting has proven useful to deliver antigens to dendritic cells (DCs) to either promote
197 vaccine-induced immune responses or anti-tumor responses, but also to induce tolerance [47].
198 A study demonstrated efficient DC targeting of the retinoic acid inducible gene I (RIG-I)
199 agonist poly-dA:dT by coupling it to a mAb against DEC-205 [48]. Administration of the
200 DEC-205-specific mAb conjugated to poly-dA:dT in combination with a model antigen
201 resulted in efficient T cell priming, suggesting that dual DC targeting of antigens and
202 adjuvants may be a means to enhance vaccine efficacy. A similar approach was described
203 recently for the CLR blood dendritic cell antigen 2 (BDCA-2), inducing tolerance in this
204 experimental setting [49]. BDCA-2 is exclusively expressed by plasmacytoid DCs (pDCs)
205 and is known to be involved in antigen capture and presentation. Antibody-mediated targeting
206 of pDCs in mice expressing human BDCA-2 led to suppression of antigen-specific $CD4^+$ T
207 cell and antibody responses mediated by regulatory T cells. These recent studies exemplify
208 that, based on the respective CLR and the immunological setting, DC targeting may be
209 employed either to augment cellular activation or to induce tolerance.
210
211 **Carrier systems for multivalent display of glycans for CLR targeting**
212 Currently, most CLR targeting strategies utilize monoclonal antibodies, whereas the use of
213 glycan-based CLR targeting approaches has only become more attractive in recent years [50,
214 51]. Glycan-based CLR targeting features several advantages when compared to antibody-
215 mediated targeting, including the lower immunogenicity of carbohydrates and the opportunity
216 to target several CLRs simultaneously. In addition, the glycan ligand density as well as the
217 spatial orientation of ligands can be manipulated allowing for the design of effective
218 multivalent binding systems [52]. Although only a limited number of carbohydrate ligands for
219 CLRs are currently known, the utility of glycan-based CLR targeting has been demonstrated
220 in several studies [53, 54].

To date, multivalent glycan presentation on glycoclusters, polymers, model antigens (proteins and peptides), liposomes, dendrimers, and nanoparticles has been employed for CLR targeting (Figure 5). Various parameters such as size, surface charge, ligand density, and conjugation methods may impact targeting efficacy [47, 55]. In a recent study, the administration of the TLR9 agonist CpG DNA, wrapped with the Dectin-1 ligand schizophyllan triggered robust type-I and type-II interferon responses [56]. This study indicates that both the choice of the CLR ligand and the mode of its presentation (in this case in a particulate fashion) have a marked effect on its immune stimulatory capacity.

Nanoparticles have become a promising platform for multivalent glycan display in CLR targeting and imaging [57]. A promising target is DC-SIGN that binds with high affinity to *N*-linked high-mannose glycan clusters on the HIV gp120 glycoprotein. Thereby, DC-SIGN is exploited by HIV in that HIV capture by DC-SIGN facilitates trans-infection of $CD4^+$ T cells via the interaction between DC-SIGN on DCs with ICAM-3 on T cells. To mimic the cluster presentation of high-mannose glycans on the viral surface, gold nanoparticles were prepared that displayed gp120-related oligomannosides [58]. The glyconanoparticles were endocytosed partially by DC-SIGN-dependent pathways, thus inhibiting DC-SIGN-mediated HIV-1 trans-infection of human T cells *in vitro*. In another study, DC-SIGN targeting was mediated by gold nanoparticles functionalized with α-fucosyl-β-alanyl amide. These nanoparticles were as effective in cell internalization as particles coated with the DC-SIGN oligosaccharide ligand Lewis-X (Le^x) [59]. Galactofuranose-coated gold nanoparticles were synthesized and evaluated regarding their ability for DC targeting and immune modulation [60]. Nanoparticles displaying galactofuranose bound to DC-SIGN on monocyte-derived DCs and enhanced the expression of DC maturation markers and pro-inflammatory cytokines. Importantly, glycan-coated nanoparticles exhibited high stability under physiological conditions such as protein-rich media or human plasma [61, 62]. The carbohydrate shell on the surface of gold nanoparticles enabled specific lectin targeting on tumor cells while avoiding nonspecific

phagocytosis by macrophages. This observation renders plasmonic nanoparticles promising tools for *in vivo* applications such as the photothermal therapy of tumors [61]. Another study used hydroxyethyl starch nanocarriers that were PEGylated and further mannosylated on the outer PEG layer [62]. Because these mannosylated nanocarriers displayed high DC targeting efficacy while exhibiting low nonspecific protein adsorption, they may be promising tools for *in vivo* applications as well. Recently, core-shell silica superparamagnetic glyconanoparticles were synthesized that bound to E- and P-selectin on endothelial cells [63]. These particles were used in a clinically relevant animal model of stroke and were found to accumulate in the brain vasculature as revealed by magnetic resonance imaging. In conclusion, glyconanoparticles may hold great potential for relevant *in vivo* applications.

In addition to nanoparticles, **liposomes** are attractive carrier systems for multivalent glycan display [64]. To date, most studies have focused on DC-SIGN targeting to deliver vaccine antigens into DCs. Liposome modification with the DC-SIGN-binding glycans Lewis-B (Le^B) and Le^x increased the binding and internalization by bone marrow-derived DCs expressing human DC-SIGN and elicited robust $CD4^+$ and $CD8^+$ T cell responses against a melanoma antigen [65]. A recent study analyzed the effect of adjuvant encapsulation into liposomes containing the DC-SIGN ligand Le^x [66]. Liposomes that had been loaded with different adjuvants were indeed endocytosed by monocyte-derived DCs in a DC-SIGN-dependent manner. In particular, the TLR4 ligand MPLA exhibited substantial adjuvant activity with regard to DC maturation and the production of pro-inflammatory cytokines and markedly enhanced cross-presentation of a melanoma antigen to $CD8^+$ T cells. This study shows that glycoliposomes constitute powerful tools in anti-tumor vaccine design that combine DC targeting specificity (by multivalent glycan display), loading of tumor antigens (for $CD8^+$ T cell cross-priming) and potent adjuvants. Similar results were obtained in a human skin explant model that was used to analyze intradermal liposome localization [67]. In this study, liposomes were efficiently taken up by $CD14^+$ dermal DCs, indicating their utility for skin

vaccine delivery. An interesting study compared targeting of the CLRs DC-SIGN, expressed by DCs, with Langerin, expressed by Langerhans cells (LCs) [68]. To this end, liposomes displaying the difucosylated oligosaccharide Lewis-Y (Le^Y), a ligand for both DC-SIGN and Langerin, were used. While Le^Y-displaying liposomes were efficiently endocytosed by DC-$SIGN^+$ DCs, no endocytosis by Langerin-expressing LCs was observed. In contrast, LCs were selectively targeted using Le^Y-modified peptides, suggesting that size, shape, and ligand density are important parameters that need to be taken into consideration when targeting different CLRs.

In recent years, **dendrimers** and **polymers** have received increased attention for multivalent display of glycans, mainly due to their unique recognition properties and the advances in manufacturing well-defined glycopolymers with controlled lengths, compositions, and architectures [69]. Most studies have used dendrimers and polymers functionalized with mannose or Lewis blood group antigens for CLR targeting. A set of poly(phosphorhydrazone) dendrimers grafted with mannose was synthesized to mimic the supramolecular structure of mannose-capped lipoarabinomannan [70]. The highest binding avidity for DC-SIGN was observed for a third-generation mannodendrimer displaying 48 trimannoside caps and a fourth-generation dendrimer bearing 96 dimannosides. These dendrimers efficiently inhibited the production of pro-inflammatory cytokines by DCs *in vitro* and were able to reduce neutrophil influx in a murine model of acute lung inflammation. In another study, polyethylene glycol-based dendrons, so-called PEGtide dendrons, were designed which contained alternating poly(ethylene glycol) and amino acid/peptide moieties and displayed different numbers of mannose residues [71]. Third-generation PEGtide dendrons containing eight mannose residues were efficient in MR-mediated macrophage targeting as revealed by confocal microscopy. Dendrimers presenting Le^x moieties were recently used for DC-SIGN-based DC targeting [72]. Indeed, Le^x dendrimers bound to DC-SIGN with high selectivity and avidity and competed with HIV gp120 binding to DC-SIGN. Interestingly, glycodendrimers

with larger molecular diameters exhibited increased DC-SIGN binding, highlighting the importance of the geometry of multivalent compounds. Besides dendrimers, polymers have also been used for DC-SIGN targeting, including multi-block glycopolymers containing mannose, glucose, and fucose moieties [73]. The binding affinities for these different glycopolymers to DC-SIGN were determined by surface plasmon resonance (SPR) measurements and revealed higher binding affinities for polymers with higher mannose content. Another carrier strategy for multivalent display of glycans in a spherical manner is the use of **fullerenes**. Mannosylated fullerenes bearing 36 mannose moieties were synthesized and evaluated for their binding to DC-SIGN [74]. These glycofullerenes were efficient inhibitors of DC-SIGN-mediated cell infection with virus particles displaying Ebola virus envelope glycoprotein (so-called pseudotyped Ebola virus), suggesting potential applications of glycofullerenes to interfere with CLR/glycan interactions.

Even more promising may be approaches where the above-mentioned strategies are combined to generate nested layers of multivalency to further increase binding avidity as seen in **glycoclusters**. An example is glyco-dendri-protein-nano-particles (with up to 1620 glycans) that exhibit diameters of up to 32 nm and are capable of mimicking pathogens both in size and surface glycosylation [75]. In T cell and DC infection studies using pseudotyped Ebola virus, these glycoclusters were efficient inhibitors of infection at picomolar concentrations.

The development of **glycomimetics** as novel CLR ligands serves as a strategy to increase the affinity of a single glycan/CLR interaction. Multivalent display of carbohydrate and non-carbohydrate glycomimetics on dendrimers or nanoparticles can be employed to further increase binding avidity. One study compared two glycomimetic antagonists of DC-SIGN, a pseudomannobioside and a linear pseudomannotrioside, regarding their binding mode and DC-SIGN clustering [76]. Promising glycomimetic DC-SIGN antagonists were also displayed on dendrimers to augment DC-SIGN binding as determined by SPR measurements [77]. Nanomolar inhibitors of DC-SIGN-based HIV trans-infection of T cells were designed by

combining a monovalent glycomimetic DC-SIGN ligand with trivalent dendrons separated by a rigid core of controlled length [78]. Notably, high-affinity non-carbohydrate glycomimetics for DC-SIGN were also developed that exhibited activity as either DC-SIGN agonists or antagonists [79].

Finally, glycan-modified **antigens** (proteins or peptides) can be used for CLR targeting on APCs. For instance, a synthetic β-(1-3)-glucan hexasaccharide was used as a Dectin-1 agonist to target skin DCs [80]. Conjugation to a model antigen and immunization of mice led to substantial antibody titers, suggesting the utility of CLR ligands as potential adjuvants for antigen delivery to skin DCs. A similar strategy was recently exploited by incorporating the β-glucan Dectin-1 ligand laminarin into a β-mannan tetanus toxoid conjugate, thus generating a tricomponent conjugate vaccine against *Candida albicans* [81]. Immunization studies in mice revealed augmented antibody responses against *C. albicans* β-mannan. Rational design of glycan ligands of CLRs as well as the optimization of the respective protein or peptide antigen can further increase targeting efficacy [82, 83]. A particularly attractive strategy is targeting multiple CLRs simultaneously to enhance targeting or to induce synergistic responses through cross-talk. A recent study analyzed the internalization properties of several CLRs in a comparative approach. Differences in intracellular routing were observed when MR, DC-SIGN, MGL, and DCIR were compared thus providing novel insights for the design of immunotherapeutic strategies based on CLR targeting [84].

Concluding remarks and future perspectives

In recent years, tremendous progress has been made in the expression of glycoproteins with defined and homogenous glycosylation patterns as well as in the synthesis of defined glycan structures. Owing to the advances in heterologous expression systems such as genetically engineered bacteria, yeasts, plants, insect cells, and mammalian cells, the production of fully humanized glycoforms can be envisaged in the future. Chemical and chemoenzymatic

syntheses of glycans as well as their multivalent display on carrier systems make it possible to study molecular interactions with lectins expressed by immune cells. Still, numerous questions regarding the application of glycans for lectin targeting in innate immunity and their utility to modulate immune responses remain open (see Outstanding Questions). To date, most studies have focused on selected lectins and carrier systems. However, comparative approaches reflecting multiple parameters such as the spatial orientation of glycan ligands, ligand density, and the backbone and geometry of the carrier are widely missing. In addition, little is known on how simultaneous targeting of multiple lectins impacts signaling and cross-talk with other pathways in innate immunity. Future studies and joint efforts combining the expertise of chemists, material scientists, (glyco)biologists, and immunologists are needed to ultimately achieve the goal of exploiting lectin targeting for the selective modulation of immune responses. Further advances in glycoengineering, carrier system design, and deciphering CLR signaling will pave the way towards exploiting CLR targeting for next-generation vaccine design, and the treatment of autoimmune diseases and cancer.

Box 1. C-type lectin receptor (CLR) engagement and signaling

Myeloid CLRs such as the Mannose receptor (MR), DC-SIGN, Dectin-1, or MGL-1 are preferentially expressed by antigen-presenting cells (APCs) and serve as pattern recognition receptors that sense pathogen-associated molecular patterns (PAMPs), but also danger-associated molecular patterns (DAMPs) released upon tissue damage or cell death [85]. CLR engagement by pathogen-derived ligands or self-ligands may lead to endocytosis and/or elicit signaling pathways in CLR-expressing APCs. The signaling cascade induced upon CLR activation is strongly affected by the intracellular motif present in the cytoplasmic domain of the CLR itself or associated CLR adaptor proteins. Depending on the presence of immunoreceptor tyrosine-based activation motifs (ITAMs) or immunoreceptor tyrosine-based inhibition motif (ITIMs) either activatory or inhibitory signals may be triggered. While CLRs

that carry an intracellular hemITAM or are associated with ITAM-bearing adaptor proteins activate the spleen tyrosine kinase (SYK), CLRs with an intracellular ITIM recruit phosphatases such as SHP-1/2 upon activation (Figure I). SYK activation by hemITAM- or ITAM-coupled CLRs induces the formation of a ternary complex involving the adaptor proteins CARD9, BCL10, and MALT1, finally resulting the in activation of the transcription factor NF-κB. Other transcription factors (NFAT, AP-1) may be activated upon CLR engagement as previously observed for Dectin-1 and DC-SIGN (Figure I). In contrast, ITIM-coupled CLRs may inhibit signaling initiated by activating CLRs and Toll-like receptors (TLRs). In summary, CLR-mediated signaling impacts APC functions such as cytokine production, the expression of co-stimulatory molecules, and subsequent T cell activation (Figure I).

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Figure Legends

Figure 1. Host Systems for Heterologous Gene Expression. Different systems, such as expression in prokaryotes (Gram-negative and Gram-positive bacteria), in eukaryotic cells (*e.g.* yeasts, insect cells, protozoa, plants, mammalian cells), and with cell-free expression systems are currently used for protein production.

Figure 2. General Features of Currently Used Protein Expression Systems. In human glycoproteins, complex, high mannose, and hybrid *N*-glycans are present (top). In contrast, *E. coli* lacks *N*-linked protein glycosylation, thus making it necessary to transfer *N*-glycosylation machinery from other bacteria such as *Campylobacter jejuni* to *E. coli*. Yeast-expressed glycoproteins display hypermannosylated *N*-glycans, whereas glycoproteins derived from insect cells (such as the well-established High Five cell line) carry high mannose *N*-glycans as well as truncated, paucimannosidic *N*-glycans. Mammalian cell lines (such as CHO cells) are able to produce glycoproteins with complex, human-like *N*-glycans but also exhibit the immunogenic α-Gal epitope as well as the non-human sialic acid *N*-glycolylneuraminic acid (Neu5Gc, bottom).

Figure 3. Protein Glycosylation Impacts Immunogenicity. Glycosylation affects protein functions such as biophysical properties and pharmacokinetics (bottom) as well as molecular recognition, and immune response (top). For instance, antibody Fc glycosylation (particularly α-1,6 core fucosylation) affects antibody-dependent cellular cytotoxicity (ADCC, top left); glycan structures may be recognized by lectin receptors on host antigen presenting cells thus impacting antigen uptake and subsequent T cell activation (top, middle); glycans may also contribute to allergic reactions (top, right).

Figure 4. Overview of Selected Groups of the C-type Lectin Receptor Superfamily. Depicted are members of the collectin (left) and selectin family (middle), and myeloid C-type lectin receptors (right).

Figure 5. Overview of Carrier Systems Used for Multivalent Glycan Display. Multivalent glycan presentation on glycoclusters, polymers, antigens (proteins and peptides), liposomes, dendrimers, and nanoparticles has been successfully employed for CLR targeting.

Figure I. C-type Lectin Receptor Engagement Impacts Antigen Presenting Cell Functions and Subsequent T Cell Activation. Myeloid CLRs recognize glycan structures through their carbohydrate recognition domain(s). The signaling pathways induced upon CLR engagement differ between CLRs and depend on their cytoplasmic signaling motifs such as an intracellular hemITAM, the association with ITAM-bearing adaptor proteins such as the FcRγ chain, or an intracellular ITIM (left). While the engagement of CLRs carrying an ITIM leads to the recruitment of phosphatases such as SHP-1 and SHP-2, hemITAM-coupled and FcRγ chain-associated CLRs activate the spleen tyrosine kinase (SYK). Downstream signaling results in the activation of transcription factors such as NF-κB, NFAT, and AP-1 in antigen presenting cells expressing the respective CLR (middle). As a consequence, CLR-mediated signaling may finally impact cytokine production, the release of T cell polarizing factors, and T cell priming (right).

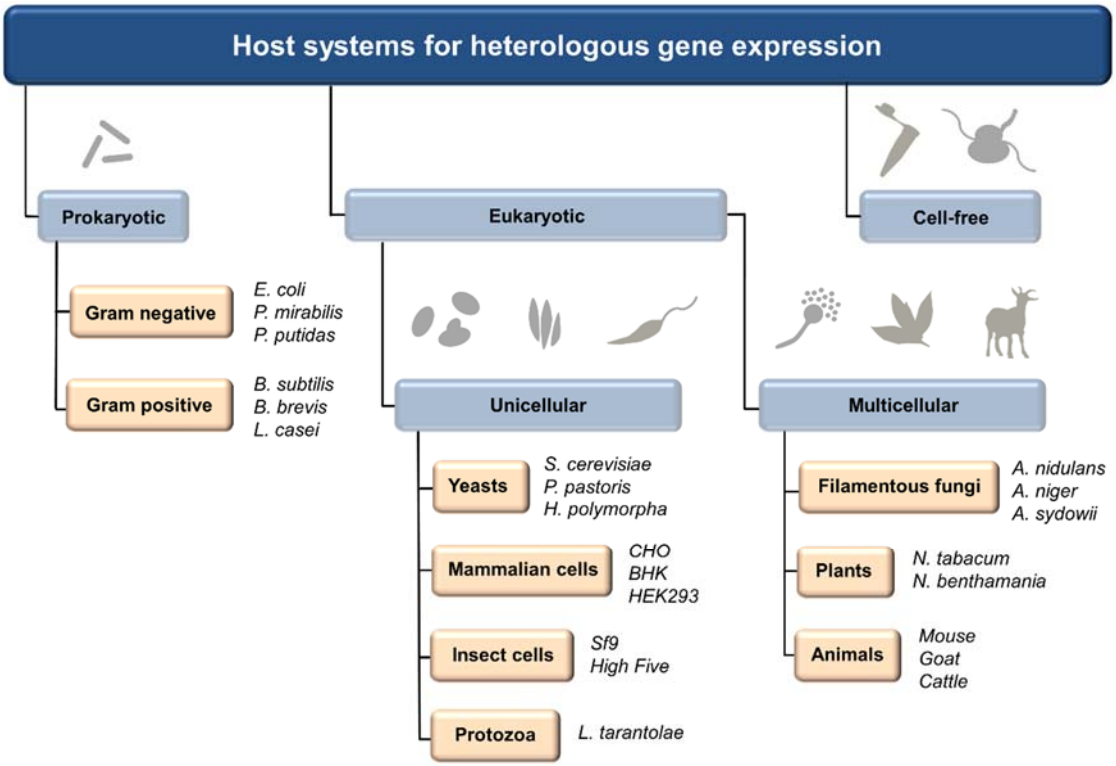

Figure 1

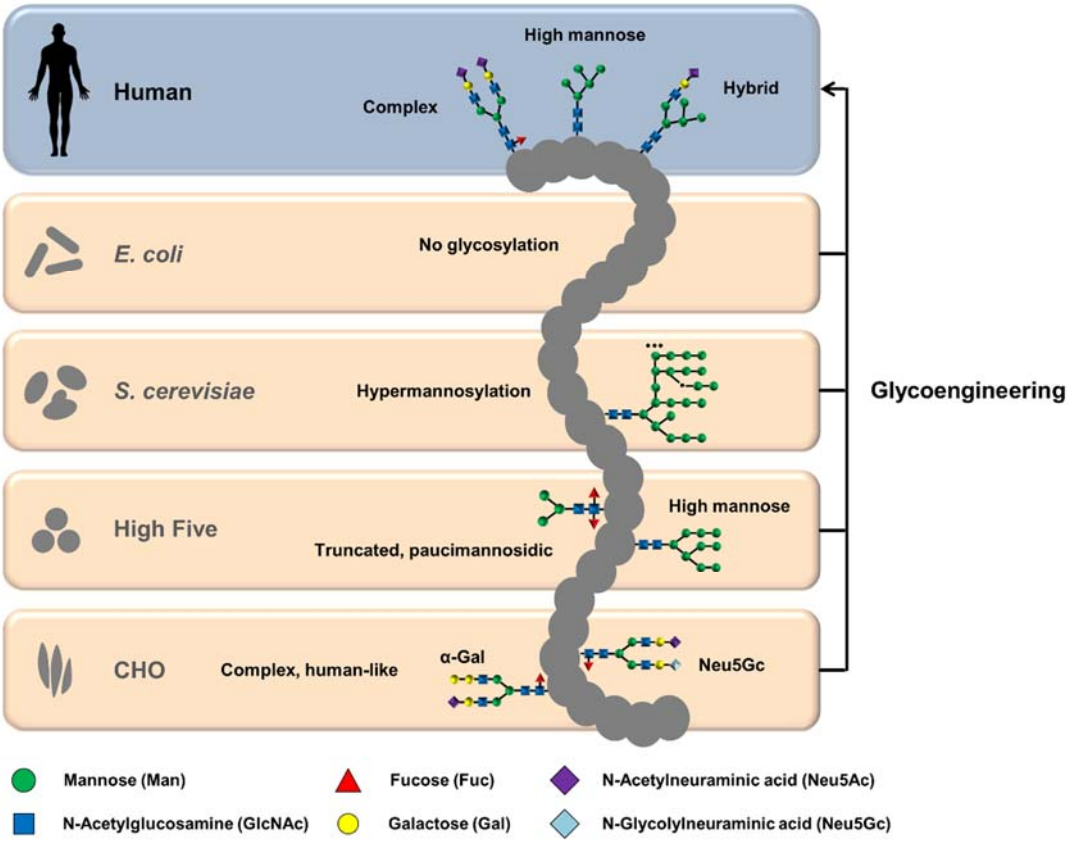

Figure 2

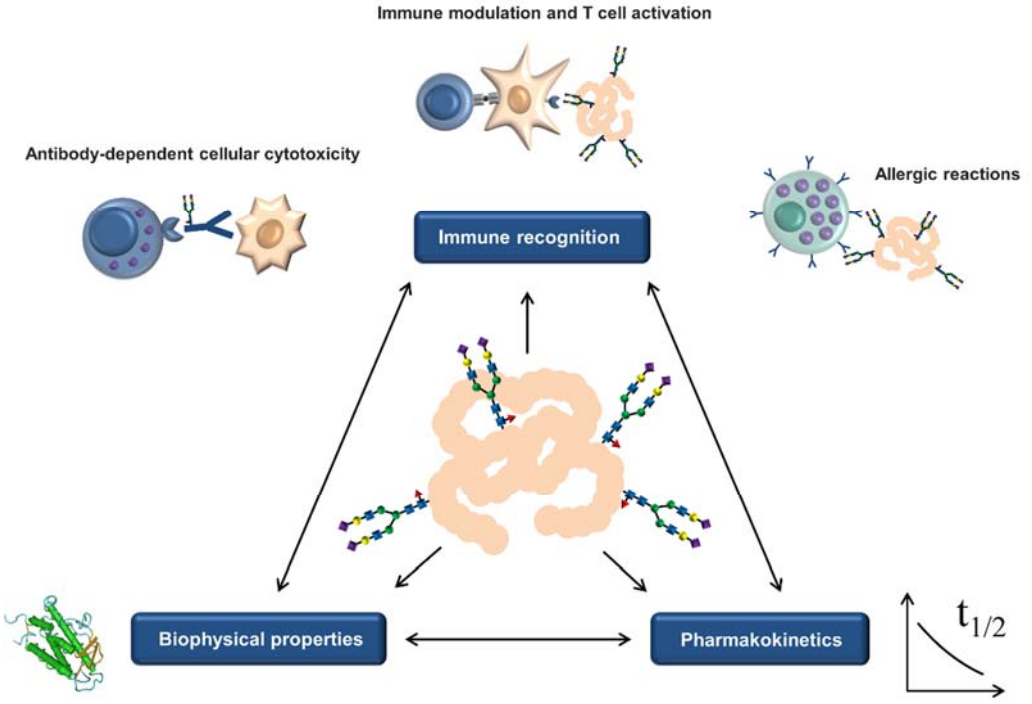

Figure 3

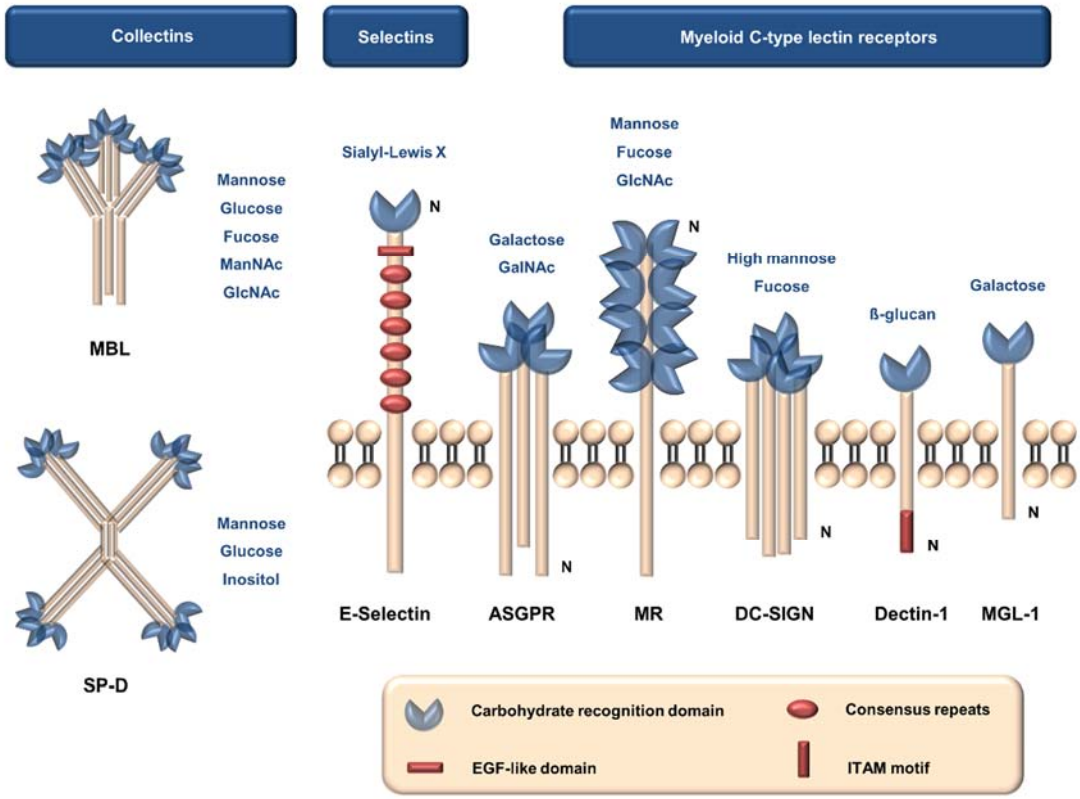

Figure 4

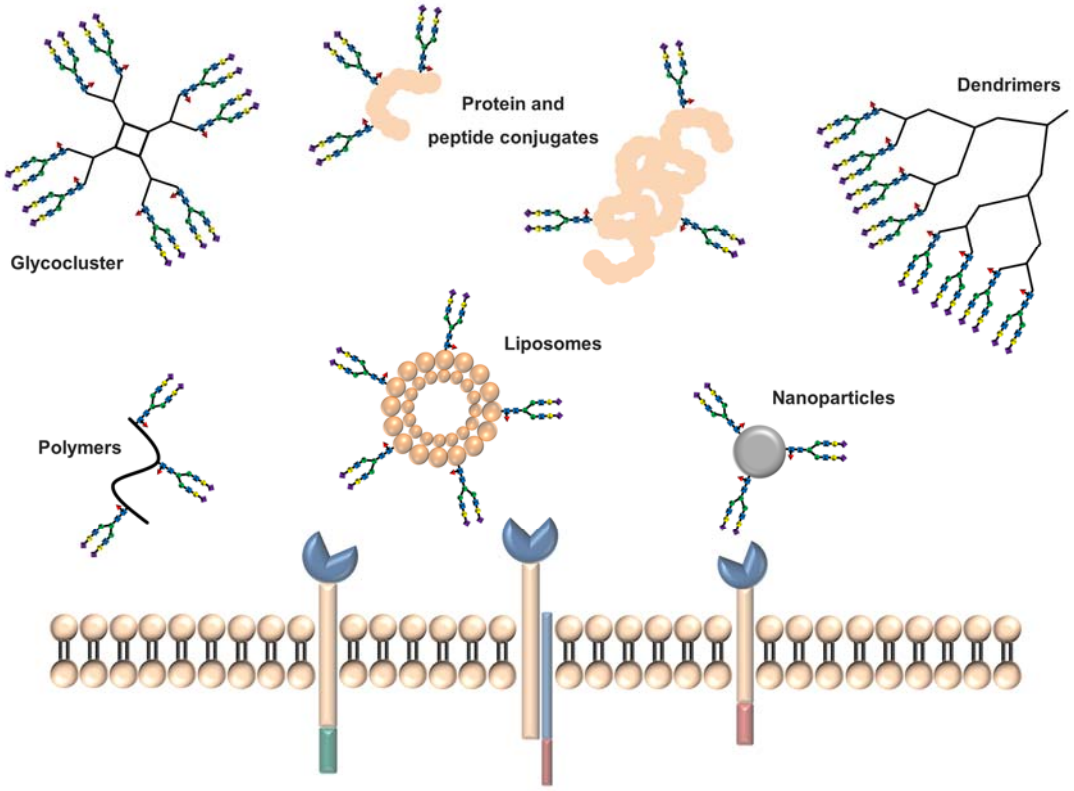

Figure 5

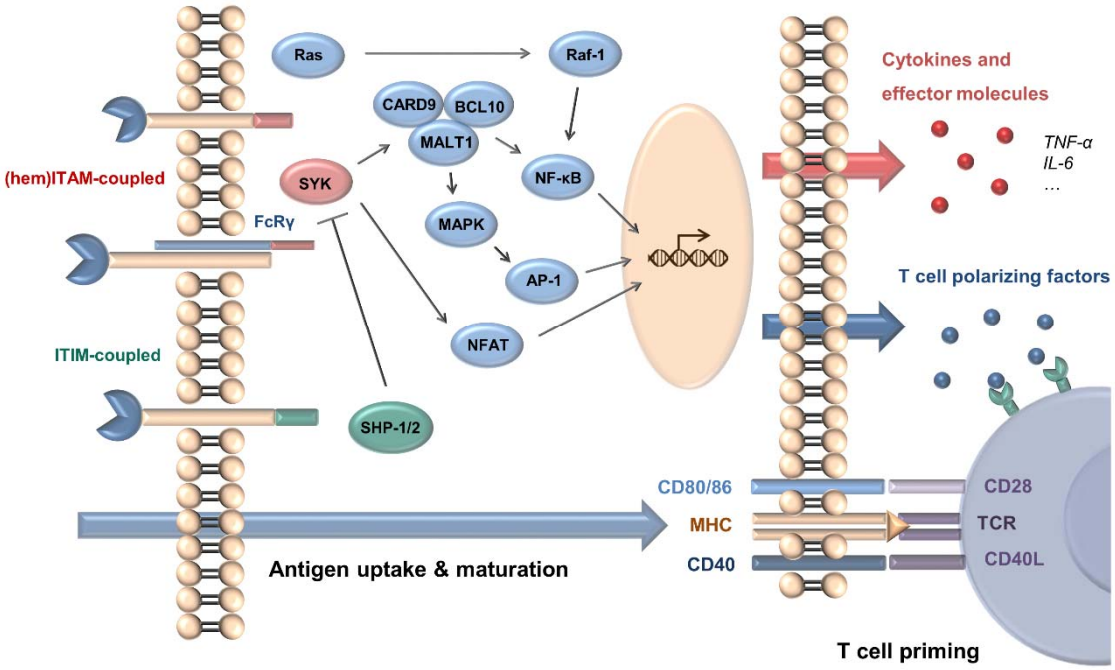

Figure I