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Targeting C-type lectin receptors with multivalent carbohydrate ligands ☆

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ABSTRACT

C-type lectin receptors (CLRs) represent a large receptor family including collectins, selectins, lymphocyte lectins, and proteoglycans. CLRs share a structurally homologous carbohydrate-recognition domain (CRD) and often bind carbohydrates in a Ca^{2+}-dependent manner. In innate immunity, CLRs serve as pattern recognition receptors (PRRs) and bind to the glycan structures of pathogens and also to self-antigens. In nature, the low affinity of CLR/carbohydrate interactions is overcome by multivalent ligand presentation at the surface of cells or pathogens. Thus, multivalency is a promising strategy for targeting CLR-expressing cells and, indeed, carbohydrate-based targeting approaches have been employed for a number of CLRs, including asialoglycoprotein receptor (ASGPR) in the liver, or DC-SIGN expressed by dendritic cells. Since CLR engagement not only mediates endocytosis but also influences intracellular signaling pathways, CLR targeting may allow for cell-specific drug delivery and also the modulation of cellular functions. Glyconanoparticles, glycodendrimers, and glycoliposomes were successfully used as tools for CLR-specific targeting. This review will discuss different approaches for multivalent CLR ligand presentation and aims to highlight how CLR targeting has been employed for cell specific drug delivery. Major emphasis is directed towards targeting of CLRs expressed by antigen-presenting cells to modulate immune responses.

Contents

1. Introduction

Targeting glycan-binding receptors has great potential for cell-specific drug and gene delivery [1,2]. Since lectin/carbohydrate interactions are characteristically weak, multivalent ligand presentation is an attractive strategy to enhance the affinity of such weak binding interactions. C-type lectin receptors (CLRs) belong to a large family of receptors that share a structurally homologous carbohydrate-recognition domain (CRD) and often bind to glycan structures in a Ca^{2+}-dependent manner [3]. Among other members, the CLR family includes the collectins, selectins, lymphocyte lectins, and proteoglycans. Because of their endocytic properties, CLRs are suitable targets for cell-specific drug delivery [4]. In the liver, the mammalian asialoglycoprotein receptor (ASGPR) was exploited as a target for gene and drug delivery because it is exclusively expressed by parenchymal hepatocytes [5].

Besides cell-specific drug delivery, CLRs may also be exploited to modulate functions of CLR-expressing cells such as endocytosis or cell

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activation. In innate immunity, CLRs are mainly expressed by antigen-presenting cells (APCs) such as dendritic cells (DCs) and macrophages. They serve as pattern-recognition receptors (PRRs) and bind to pathogen-associated molecular patterns (PAMPs) [6] but may also sense self-antigens released by tissue damage or dead cells [7]. Thus, myeloid CLRs such as DC-SIGN, mannose receptor (MR), DEC-205 and others are crucial to initiate immune responses against a number of pathogens including bacteria, viruses, parasites, and fungi (Fig. 1A). Some CLRs serve solely as phagocytic receptors whereas others activate signaling pathways in the CLR-expressing cell [8]. In the latter case, CLR engagement may induce the activation of tyrosine kinases such as Syk through classical immunoreceptor tyrosine-based activation motifs (ITAMs) or hemITAMs (Fig. 1B). In contrast, CLRs such as the DC immunoreceptor (DCIR) or the myeloid C-type lectin-like receptor (MICL) bear intracellular immunoreceptor tyrosine-based inhibitory motifs (ITIMs). As a consequence, phosphatases such as SHP-1 or SHP-2 are recruited to these ITIM-bearing CLRs which may lead to down-regulation of cellular responses. Thus, targeting myeloid CLRs expressed by APCs is not only useful for cell-specific delivery of drugs, genes or vaccine antigens, but may also serve as a means to either stimulate or dampen APC functions depending on the respective CLR.

For a long time, antibody-mediated CLR targeting has been the preferred strategy for antigen delivery and the activation of DCs in vivo [9]. Initial work focused on DEC-205, a CLR abundantly expressed in lymphoid tissues. Coupling of the model antigen ovalbumin (OVA) to a DEC-205-specific antibody resulted in the induction of antigen-specific $CD8^+$ T cell tolerance [10] or – in case of targeting maturing DCs via DEC-205 – efficient immunity in vivo [11]. A number of other CLRs have since been successfully targeted by CLR-specific antibodies indicating that this strategy is useful for cross-priming of $CD8^+$ T cells [12], tumor therapy [13], or targeted vaccine delivery [14]. In recent years, however, glycan-based targeting approaches have gained increasing attention [15]. Despite the limitation that glycan ligands need to be presented in a multivalent fashion to allow for efficient targeting, this strategy is promising because of the tunable properties of the glycan-displaying carrier systems [16,17]. Compared to antibody-mediated targeting, the spatial orientation of displayed carbohydrate CLR ligands can be varied more easily according to the distances between receptor binding sites. In addition, the rigidity of the carrier as well as the length of the spacer covalently linking carrier and carbohydrate ligands are easily modifiable, thus allowing the further enhancement of the targeting efficacy [18].

Nanoparticles can serve as vehicles for the delivery of drugs, genetic materials, proteins, and small molecules [19]. In addition, nanoparticles can be used to investigate the interaction of CLRs with their cognate ligands as they are suitable for imaging and sensing functions as well [20,21]. Indeed, particulate carriers such as polymeric nanoparticles were utilized for antigen targeting to DCs as they allow antigens and adjuvants to be delivered into the same APC [22]. Studies in which nanoparticles were functionalized with CLR-specific antibodies revealed that the target ligand density on the particles plays a crucial role in DC activation, thus it is important for the design of efficient vaccine delivery systems [23]. This renders multivalent presentation of glycan ligands a promising strategy because inter-distance spacing between glycan ligands may be optimized to allow for efficient targeting of one specific CLR or to facilitate CLR clustering.

Besides carbohydrate-functionalized nanoparticles, glycodendrimers and polymers as well as glycoliposomes were successfully employed for CLR-specific targeting [24,25]. For instance, oligomannose-coated liposomes served as antigen-delivery vehicles into DCs and macrophages leading to induction of a Th1-specific immune response and efficient cross-priming of $CD8^+$ T cells [26].

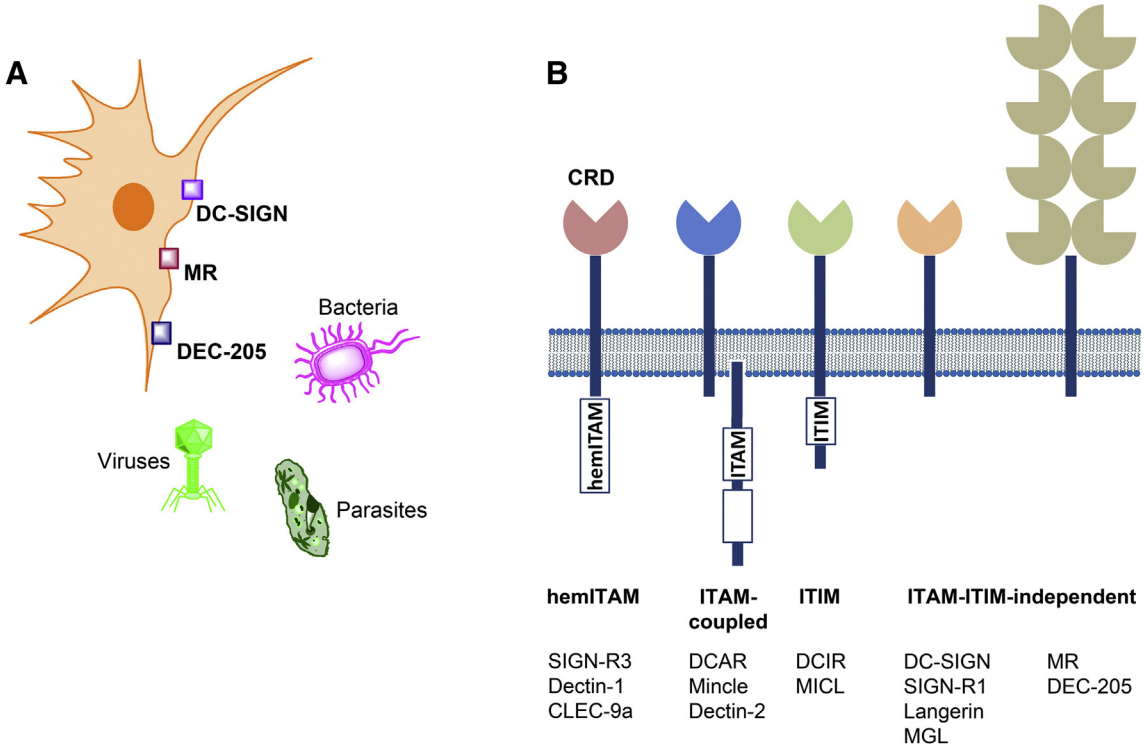

Fig. 1. Myeloid C-type lectin receptors (CLRs) in innate immunity. A, In innate immunity, CLRs such as DC-SIGN, mannose receptor (MR), or DEC-205 serve as pattern-recognition receptors and bind to glycan structures present on pathogens such as bacteria, viruses, or parasites. B, Myeloid CLRs share one or more homologous carbohydrate-recognition domains (CRDs) that bind to carbohydrates often in a Ca^{2+}-dependent manner. The signaling pathway elicited upon CLR engagement depends on their cytoplasmic signaling motifs. Some CLRs contain an intracellular hemITAM or are associated with ITAM-bearing adaptor proteins leading to the activation of the tyrosine kinase Syk. In contrast, other CLRs contain an intracellular ITIM that induces the recruitment of phosphatases such as SHP-1 and SHP-2. Another group of myeloid CLRs including DC-SIGN or MR does not possess intracellular signaling motifs, thus signaling is independent of Syk or phosphatases.

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This review will discuss different approaches for multivalent CLR ligand presentation and it will also highlight how CLR targeting has been utilized for cell-specific drug delivery and immune modulation. We will mainly focus on two classes of CLRs: 1) ASGPR for drug/gene delivery to the liver, and 2) DC-SIGN, MR, and other CLRs expressed by macrophages and DCs for APC targeting and immunomodulation.

2. Drug/gene delivery via asialoglycoprotein receptor (ASGPR) on hepatocytes

Hepatic lectin receptors are capable of mediating the endocytosis of bound ligands. Following recognition, bound ligands are internalized via coated pits and the complex is then released into endosomal compartments whereupon the lectin receptor is recycled to the cell surface [27]. Thus, hepatic lectin receptors represent promising targets to be exploited for liver-specific drug and gene delivery. A major focus has centered on the ASGPR, a type II transmembrane protein regulating the homeostasis of serum glycoprotein levels by binding and uptake of desialylated (i.e. galactose-terminated) glycoproteins [28]. The human ASGPR consists of two subunits, H1 and H2, with the H1 subunit mediating Ca^{2+}-dependent galactose/GalNAc recognition. Since ASGPR is exclusively expressed by parenchymal hepatocytes, it is an attractive target for liver-specific drug delivery [29].

A number of studies describe the use of protamine-asialofetuin lipoplexes for DNA transfection into hepatocytes [30]. Uptake may be inhibited competitively in the presence of asialofetuin indicating ASGPR-mediated endocytosis. One study reported the synthesis of cholesterylated thiogalactosides which led to the formulation of lipid–polycation–DNA complexes composed of galactosylated cationic liposomes, protamine sulfate and plasmid DNA [31]. In a more recent study, liposomes incorporated with synthetic glycolipids containing a terminal GalNAc residue were shown to be internalized into hepatocytes by ASGPR-mediated endocytosis [32].

Galactosylated poly(ethylene glycol)-chitosan-graft-polyethylenimines were also prepared as hepatocyte-targeting gene carriers [33]. Indeed, galactosylated PEG-chitosan-graft-PEI complexes with DNA were superior to PEI after intravenous injection in mice. Furthermore, galactosylated chitosan derivatives were used for hepatocyte-specific imaging. Intravenous injection of ^{99m}Tc radiolabeled hydrazinonicotinamide-galactosylated chitosan led to selective accumulation in the liver that could be inhibited by co-injection of free galactose [34]. As a cationic, polymer-based carrier system, galactosylated N-2-hydroxypropyl methacrylamide-b-N-3-guanidinopropyl methacrylamide (HPMA-b-GPMA) block copolymers were developed [35]. Synthesis of the block polymers was performed by aqueous reversible addition-fragmentation chain transfer (RAFT) polymerization of HPMA and N-3-aminopropyl methacrylamide (APMA). Subsequent galactosylation and guanidinylation enabled the complexation with plasmid DNA for hepatocyte targeting. The resulting polymer/DNA complexes displayed lower cytotoxicity compared to PEI and exhibited specificity for hepatocytes suggesting ASGPR-mediated transfection.

Carbohydrate-functionalized nanoparticles represent useful tools for hepatocyte-specific targeting and imaging. An early study involved the use of poly(L-lactic acid) (PLA) nanoparticles that were coated with a galactose-carrying polymer and loaded with *all trans*-retinoic acid (RA) to study RA-mediated effects in hepatocytes [36]. Besides PLA nanoparticles, super-paramagnetic iron oxide nanoparticles (SPIOs) were used for hepatocyte targeting [37]. Recently, PEGylated quantum dots (QDs) that were capped with galactose or galactosamine were synthesized [38]. These QDs served as a useful tool for targeting hepatocytes in vitro and for imaging liver-specific delivery in vivo.

To mimic the trivalent binding by the ASGPR, a series of fluorescent ligands was synthesized that contained three non-reducing β-Gal or GalNAc moieties linked to flexible spacers to optimize the spatial interaction with the binding site of the ASGPR [39]. Selective endocytosis into the HepG2 hepatocyte cell line indicated ASGPR-mediated uptake. Using a nitriloacetic acid (NTA) derivative of L-lysine as a scaffold, tri- and hexavalent structures were prepared displaying GalNAc or lactose terminals [40]. High affinities for hepatocytes were observed for the trivalent GalNAc glycosides as well as the hexavalent lactoside. Radiolabeling of the hexavalent lactoside with ^{111}In allowed for in vivo imaging. Indeed, upon intravenous infusion of the ^{111}In-tagged hexavalent lactoside, liver-specific accumulation of radioactivity was detected.

In conclusion, multivalent carbohydrate ligand display is a viable approach for ASGPR-specific targeting. The ability of the ASGPR to operate as a multifunctional receptor engaged in the endocytic removal and elimination of apoptotic cells and other cellular remnants emphasizes its importance as a key target to facilitate efficient clearance via multivalent targeting. Hence, carbohydrate-based hepatocyte targeting can be employed as a less immunogenic, safer and cheaper alternative to viral gene therapy. In addition, ASGPR-mediated drug delivery to the liver also represents a therapeutic strategy for the treatment of hepatocellular carcinoma.

3. Targeting C-type lectin receptors to shape immune responses

While ASGPR targeting on hepatocytes is a means for liver-specific drug or gene delivery, targeting myeloid CLRs in innate immunity offers the additional advantage of shaping immune responses (Fig. 2). CLR targeting by CLR-specific antibodies or multivalent CLR ligands leads to internalization into the APC whereupon the cargo (e.g. a vaccine antigen) is released and processed in endosomal compartments. Antigen-derived peptide fragments are then presented to T cells by major histocompatibility complex (MHC) molecules expressed by APCs. In addition to uptake, CLR engagement may induce signaling pathways in APCs resulting in the expression of co-stimulatory molecules CD80/CD86 as well as the production of cytokines. Besides MHC-presented peptides (signal 1), T cells require secondary co-stimulatory signaling mediated by the interaction of CD80/CD86 with CD28 on the T cells to become activated. The cytokine pattern released by APCs influences the initiated T cell response. For instance, varying fates exist for a naïve $CD4^+$ T cell upon activation. Cells may differentiate into Th1 cells for example, destined to release IFN-γ as the major effector cytokine contributing to macrophage activation. Alternatively, $CD4^+$ T cells may differentiate into Th2 cells required for B cell activation to provoke a humoral immune response, or Th17 cells involved in eliciting pro-inflammatory responses.

In conclusion, interference at the onset or very early stages during APC activation through CLR engagement may indeed have a tremendous impact on initiated T cell responses. Thus, CLR targeting is a means to shape adaptive immune responses which may be of fundamental importance in relation to vaccination, tolerance induction, or tumor therapy. The following aims to discuss approaches for targeting DC/macrophages via DC-SIGN, mannose receptor, and other CLRs.

3.1. Dendritic cell targeting via DC-SIGN

DC-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), a type II transmembrane receptor, is an attractive target for DC-specific antigen delivery [41]. It is mainly expressed by myeloid DCs, but it is also expressed by dermal, interstitial and monocyte-derived DCs. DC-SIGN recognizes highly glycosylated proteins present on the surface of pathogens which results in internalization, processing and presentation of pathogen-derived antigens. However, DC-SIGN also interacts with endogenous ligands such as the intercellular adhesion molecules (ICAM)-2 and ICAM-3 [42]. DC-SIGN contains a carbohydrate recognition domain that binds to high-mannose and fucose-terminated glycan structures as well as Lewis-type blood antigens in a Ca^{2+}-dependent manner [15]. In addition, DC-SIGN contains a flexible neck region, a

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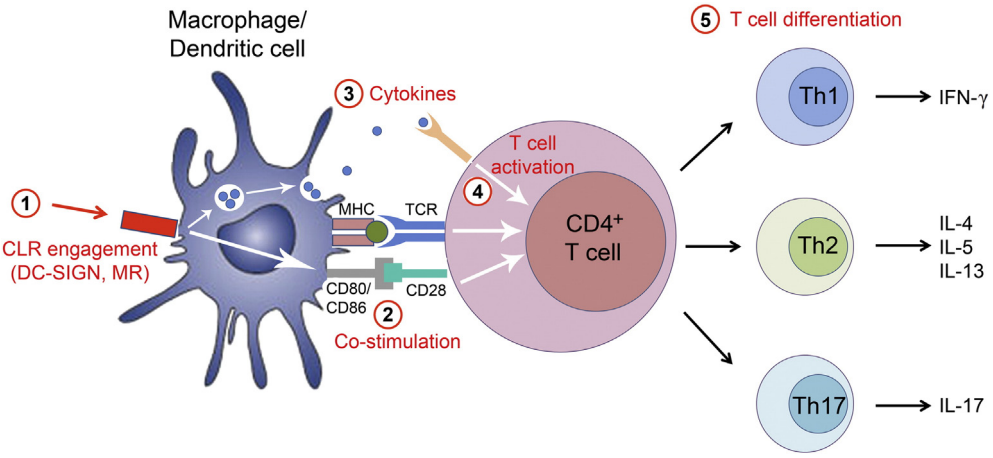

Fig. 2. CLR targeting as a means to shape an initiated immune response. Engagement of myeloid CLRs in innate immunity such as DC-SIGN or MR leads to receptor-mediated endocytosis (1). Thus, CLR targeting with CLR-specific antibodies or multivalent carbohydrate ligands can be used for cell-specific drug or gene delivery. If an antigen is targeted to an antigen-presenting cell (APC), the antigen will be internalized, processed and presented by major histocompatibility complex (MHC) molecules to T cells. In addition, signaling pathways may be provoked in APCs that lead to the expression of the co-stimulatory molecules CD80/CD86 (2) as well as the production of cytokines (3). T cells recognize MHC-presented peptides through their T cell receptor (TCR) and become activated in the presence of a secondary, co-stimulatory signal mediated via the interaction of CD80/CD86 with CD28 expressed by the T cell (4). Cytokines released by APCs influence T cell differentiation and T cell effector functions (5). For instance, naïve $CD4^+$ T cells can differentiate into Th1, Th2, or Th17 cells with each $CD4^+$ T cell subset having specific effector functions. Since CLR engagement strongly impacts the process of T cell activation, CLR targeting is a means to shape adaptive immune responses.

transmembrane region and a cytoplasmic tail that incorporates recycling and internalization motifs [43]. Though DC-SIGN was reported to be essential for carbohydrate recognition for a number of pathogens, it is also exploited by certain pathogens such as HIV and hepatitis C virus to promote infection [44,45]. In several studies, DC-SIGN-specific antibodies were used for DC targeting which were either coupled with antigens directly or bio-conjugated in a multivalent fashion on the surface of nanoparticles [46,47]. The expression of DC-SIGN in the form of tetramers at the surface of myeloid DCs has sparked much interest in DC-SIGN targeting using multivalent glycan ligand display (Fig. 3). Such targeting strategies are of particular interest with regard to cancer vaccines in order to induce tumor-specific T cells. Modification of the melanoma differentiation antigen gp100 with DC-SIGN-interacting glycans led to enhanced antigen presentation to gp100-specific $CD4^+$ T cells [48]. In another study, a high-mannose *N*-glycan prepared from affinity-fractionated soybean agglutinin was used for DC-SIGN targeting [49]. After release of the *N*-glycan structure and its subsequent conjugation to a polyacridine peptide, plasmid DNA binding was mediated by polyintercalation and ionic binding interactions. Flow cytometry revealed that high-mannose *N*-glycan modification induced a 100-fold increased uptake into DC-SIGN transfected cell lines over untransfected cells.

Since DC-SIGN is a major receptor for HIV and promotes *trans*-infection of human T cells, the multivalent display of *N*-linked high mannose glycans of the HIV gp120 is an interesting approach to inhibit HIV infection of cells. Gold glyconanoparticles represent a powerful tool for the polyvalent display of biologically relevant oligosaccharides with differing densities [50]. To inhibit DC-SIGN-mediated HIV infection of cells, water-soluble gold nanoparticles presenting truncated (oligo) mannosides of the high-mannose HIV undecasaccharide $Man_9GlcNAc_2$ were synthesized [51]. Indeed, surface plasmon resonance (SPR) revealed that these glyconanoparticles inhibited DC-SIGN binding to gp120 from the micro- to the nanomolar range and were efficient inhibitors of DC-SIGN-mediated *trans*-infection of human T cells [51]. Uptake studies into lymphoma cells and monocyte-derived DCs indicated that the oligomannoside-coated nanoparticles were efficiently endocytosed by DC-SIGN-dependent and -independent pathways and partially co-localized in early endosomes [52] (Fig. 3A).

Glycodendrimers are useful tools for multivalent CLR targeting. A glycodendrimer displaying 32 mannose residues was reported to bind to DC-SIGN in the sub-micromolar range and inhibited DC-SIGN binding to HIV gp120 [53]. In another study, multivalent presentation of oligomannoses in high density indicated specific binding to DC-SIGN and the broadly neutralizing antibody 2G12 binding to a conserved cluster of oligomannose glycans on the HIV gp120 glycoprotein [54]. The dendrimeric display led to an inhibition of gp120 binding to the 2G12 antibody as well as to recombinant DC-SIGN in the nanomolar range. Recently, poly(amido amine) dendrimers conjugated to the DC-SIGN ligand Le^b were employed to mediate cell internalization, lysosomal delivery, cytokine production, and antigen-specific T cell proliferation [55]. Interestingly, a glycan density of 16 to 32 glycan units per dendrimer was found to be optimal for uptake as well as antigen presentation. Linear pseudodi- and pseudotrisaccharides were presented in a multivalent fashion on polyester dendrons [56]. Competition experiments with Ebola pseudotyped viral particles indicated that the dendrimers were potent inhibitors of viral entry due to blocking of the DC-SIGN receptor. In another study, mannose- and fucose-functionalized glycodendrons were synthesized by Cu(I)-catalyzed azide-alkyne cycloaddition [57]. Fluorescent labeling of the glycodendrons allowed for tracking of their intracellular processing upon endocytosis. Co-localization experiments indicated that both mannosylated and fucosylated dendrons were routed to lysosomes. Recently, this approach was extended by employing nested layers of multivalency for the generation of so-called glyco-dendri-protein-nanoparticles [58] (Fig. 3B). This strategy enabled the display of up to 1620 glycans and in a model of Ebola infection of T cells and DCs, the clustered glycan presentation inhibited infection at picomolar concentrations.

Fullerenes were employed for the multivalent display of glycans in a spherical manner. Glycofullerenes bearing 36 mannose moieties were prepared to serve as binders to DC-SIGN [59] (Fig. 3C). In a competition experiment with pseudotyped Ebola virus particles, these glycoconjugates served as efficient inhibitors indicating that a fullerene-based presentation of CLR ligands might be a promising approach.

Furthermore, glycoliposomes were used for antigen targeting to DCs to enhance T cell responses. For DC-SIGN-specific targeting, liposomes were modified with Le^b or Le^x ligands [60]. Glycan modification of the liposomes induced endocytosis by bone marrow-derived DCs expressing human DC-SIGN and led to efficient $CD4^+$ and $CD8^+$ T cell priming. Encapsulation of the MART-1 melanoma antigen led to robust tumor antigen-specific $CD8^+$ T cell responses indicating

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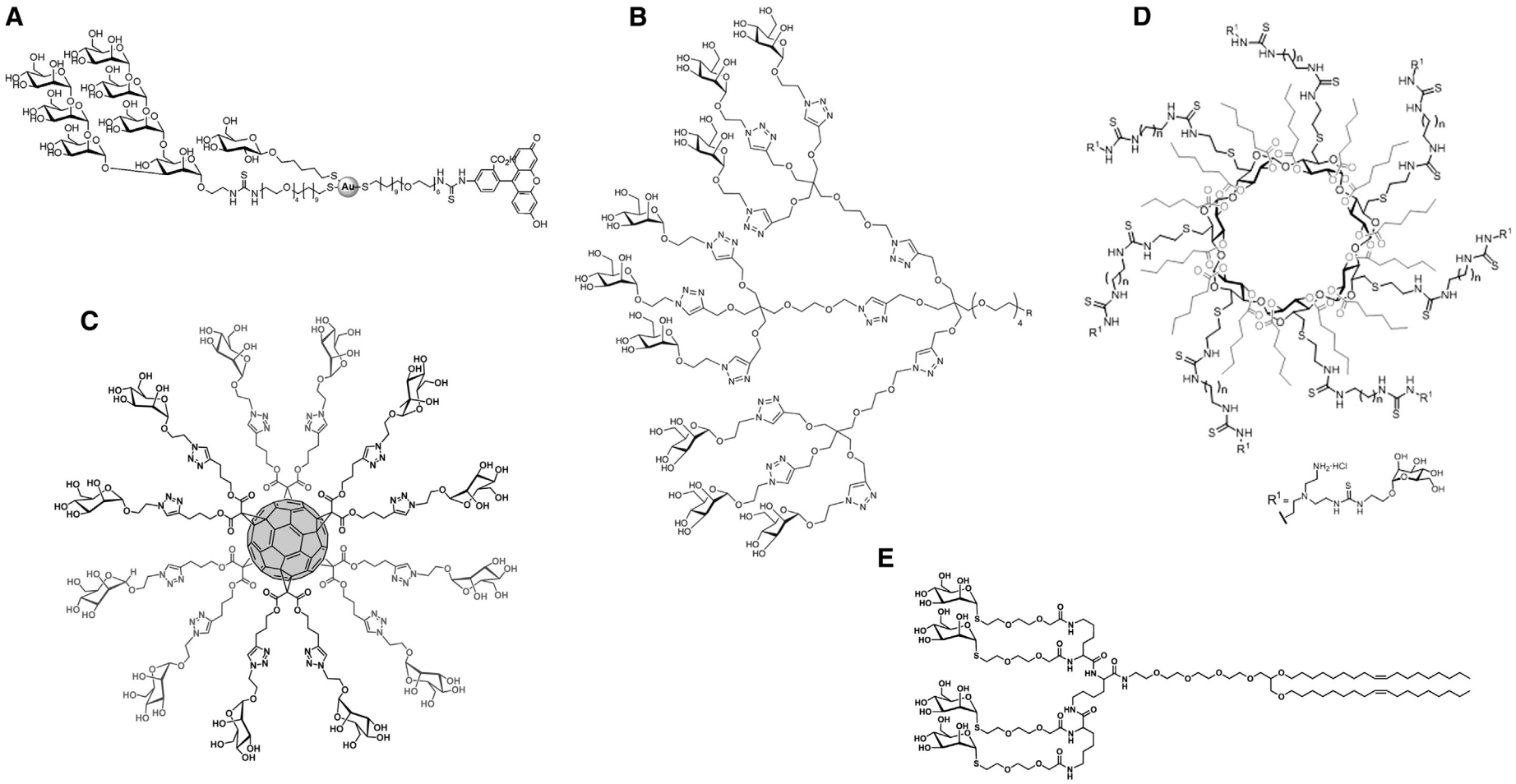

Fig. 3. Multivalent ligand presentation for targeting DC-SIGN and MR. CLRs such as DC-SIGN or MR usually display low affinities for their carbohydrate ligands, thus multivalent ligand presentation is a means to overcome these low affinities. A number of different approaches were used for CLR targeting including glyconanoparticles (A), glycodendrimers (B), glycofullerenes (C), glycoclusters (D), and glycolipids, incorporated into liposomes (E). A, Fluorescent gold nanoparticles were used to display multiple copies of structural motifs of the *N*-linked high-mannose glycan of HIV gp120 as efficient binders of DC-SIGN [52]. B, Mannose-terminated glycondendrons that were further used in the assembly of highly valent virus-like glycondri-nanoparticles to inhibit Ebola virus infection of DCs were synthesized [58]. C, Glycofullerenes presenting mannose residues were used to inhibit DC-SIGN-dependent cell infection by pseudotyped viral particles [59]. D, Multivalent polycationic glyco-amphiphilic cyclodextrins were prepared and used for targeting MR in macrophages [97]. E, Multibranched mannosylated lipids were prepared and incorporated into liposomes to allow for MR-mediated endocytosis by monocyte-derived DCs [117].

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that glycoliposome-based DC-SIGN targeting might be useful for the design of cancer vaccines. Interestingly, the formulation seems to play an important role as revealed by a comparison between PEGylated and non-PEGylated glycoliposomes where PEGylation decreased the DC targeting efficiency [61]. The fact that DC-SIGN is expressed by human, but not by murine DCs rendered investigations in relevant mouse models difficult. However, the generation of human DC-SIGN transgenic mice that express DC-SIGN under the control of the CD11c promoter now allows for analyzing DC-SIGN targeting in vivo [62]. Indeed, in human DC-SIGN transgenic mice, Le^b or Le^x modified OVA was efficiently targeted to DCs and led to efficient $CD4^+$ T cell priming as well as cross-presentation [63].

To increase DC-SIGN targeting specificity compared to canonical carbohydrate ligands, glycomimetic compounds were synthesized and were further combined with dendritic platforms to obtain novel DC-SIGN antagonists displaying strong binding affinities [64–68]. In an elegant approach based on high-throughput fluorescence-based competition assay, non-carbohydrate small molecule inhibitors were discovered with IC_{50} values in the low micromolar range [69]. The natural product shikimic acid as well as quinoxalinones were used as scaffolds to design glycomimetics acting as mannoside or fucoside surrogates [70–72]. Indeed, one glycomimetic compound was demonstrated to occupy the same carbohydrate-binding site of DC-SIGN as saccharide ligands and could be used not only as a DC-SIGN antagonist, but also as an agonist [73]. These studies indicate that glycomimetic approaches are useful for targeting DC-SIGN as well as other CLRs.

While substantial progress has been made in DC-SIGN-mediated targeting using glycan-modified carrier systems, there are still a number of challenges. Though the different carrier systems proved to be specific for DC-SIGN targeting in model systems, there is limited knowledge of the advantages and drawbacks of the different carriers in for a given in vitro or in vivo model. Thus, a more detailed comparison between the carrier systems regarding their targeting efficacy is needed. Consequently, future studies should systematically address the impact of parameters such as the nature of the carrier system (rigid vs. flexible backbones), spacer design and length, and ligand density on targeting specificity.

3.2. Dendritic cell/macrophage targeting via mannose receptor (MR)

The CLR mannose receptor (MR) is a type I transmembrane receptor that is predominantly expressed on the surface of macrophages and DCs [74]. The MR contains eight CRDs, a cysteine rich domain, a fibronectin type II domain, and a short cytoplasmic tail lacking classical signaling motifs [75]. The MR recognizes not only mannose-terminated glycans, but also fucose-terminated glycans on the surface of pathogens, thus it is capable of sensing bacterial, viral, and fungal ligands, and also endogenous ligands [76]. One function of the MR is the regulation of serum glycoprotein homeostasis [77]. Recently, a crucial role for MR in antigen cross-presentation was reported [78].

Mannosylation of antigens was reported as a successful strategy for specific targeting of macrophages and DCs. A number of studies focused on glycan-modification of tumor antigens to increase immunogenicity and to induce potent tumor-specific $CD8^+$ T cell responses. Modification of the human tumor antigen MUC1 with mannan under oxidizing conditions induced tumor-specific $CD8^+$ T cell responses in immunized mice and led to complete protection whereas reduced mannan-MUC1 conjugates induced poor protection [79]. Conjugation of MUC1 to oxidized mannan also elicited strong cellular immune responses, namely IFN-γ production, and an IgG2a-dominated antibody response [80]. It was also shown that oxidized mannan-MUC1 binds to the MR leading to efficient internalization and cross-presentation thus mediating potent antigen-specific $CD8^+$ T cell responses [81]. In a clinical study in early-stage breast cancer patients, tumor immunotherapy with oxidized mannan-MUC1 was beneficial indicating that glycan-modified MUC1-based cancer vaccines have great potential [82]. In addition to mannan-based protein delivery to APCs, this strategy was also successfully employed for receptor-mediated gene delivery [83]. Immunization of mice with OVA-encoding DNA complexed to oxidized or reduced mannan-poly-L-lysine provoked robust T cell and antibody responses highlighting the potential of mannan-based targeting for DNA cancer vaccines.

MR targeting by two glycan ligands, 3-sulfo-Le^a and tri-GlcNAc, conjugated to OVA led to an increased cell uptake of the neo-glycoconjugates compared to unmodified OVA and resulted in enhanced cross-presentation [84]. In DC/T cell co-cultivation assays, the neo-glycoconjugates elicited a higher frequency of IFN-γ producing T cells indicating a Th1-dominated cytokine response.

Mannosylated peptides might be useful for antigen-specific T cell modulation and tolerance induction in vivo. In a murine model of multiple sclerosis, experimental autoimmune encephalomyelitis (EAE), immunization with a mannosylated epitope of the proteolipid protein did not induce disease but led to antigen-specific tolerance [85]. In an adoptive transfer system, immunization of mice with mannosylated OVA peptide in complete adjuvant led to normal proliferation of OVA-specific $CD4^+$ T cells but only limited effector function such as marginal IFN-γ production, low IgG2a levels in serum and reduced delayed-type hypersensitivity (DTH) responses [86]. Thus, though T cells proliferated normally they exhibited reduced Th1 effector functions.

More complex high-mannose glycans were used for macrophage and DC targeting to serve as vaccine adjuvants. Coupling of synthetic complex oligosaccharides such as a high-mannose nonasaccharide to the model antigen OVA led to markedly enhanced antigen presentation and cross-presentation by murine DCs [87]. Thus, carbohydrate-based targeting of lectin receptors expressed by DCs is a powerful strategy to enhance antigen presentation and T cell activation. Another example is phosphatidylinositol mannosides (PIMs) which are present in the cell wall of *Mycobacterium tuberculosis* as part of the lipoarabinomannans (LAMs) or mannan-capped LAMs and were reported to bind to the MR and DC-SIGN [88]. PIM lipids served as efficient inducers of pro-inflammatory cytokines in DCs. Inclusion of the model antigen OVA in palmitoyl-phosphatidylinositol dimannoside liposomes induced efficient cross-presentation by DCs [89]. Even the glycan moiety of the PIMs lacking the lipid part is capable of inducing potent cellular and humoral immune responses. Coupling of synthetic PIM glycans to model antigens led to marked antigen-specific immune responses in immunized mice indicating that these high-mannose glycans can serve as efficient immune stimulators and adjuvants [90].

In addition to chemical modification of protein antigens, different expression systems were used that provide a natural mannosylation for MR targeting. Antigen expression in fungi such as the yeast *Pichia pastoris* resulted in *N*- and *O*-linked mannosylation [91]. Compared to unglycosylated OVA expressed in *Escherichia coli*, yeast-derived OVA induced more potent OVA-specific $CD4^+$ T cell proliferation indicating that the expression system is important to provide an immunogenic antigen glycosylation. Consistently, this was recently shown for a cell line-produced influenza virus where differential glycosylation of the major antigen hemagglutinin markedly affected T cell activation and cytokine production in vitro and also impacted hemagglutinin-specific antibody production in vivo [92].

As an alternative to the conventional mannosylation of antigens, polyamidoamine (PAMAM) dendrimers were utilized for mannose-based antigen delivery [93]. Mannosylated dendrimer OVA induced efficient DC maturation and led to substantial OVA-specific T cell responses in vitro. Strong antibody production and OVA-specific T cell responses were observed upon immunization of mice with the dendrimer constructs. Prophylactic immunization of mice in an OVA-specific tumor model resulted in reduced tumor growth indicating that mannosylated dendrimers represent promising vaccine carriers. Recently, novel synthetic approaches to fluorescent, carbohydrate-functionalized dendrimers were reported which may be applied for

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lectin sensing and imaging [94,95]. A supramolecular approach for targeting mannose-binding lectins was described [96]. The multivalent conjugates consist of a fluorescent ruthenium(II) core surrounded by a heptamannosylated β-cyclodextrin scaffold. These multivalent sensors allowed for the investigation of cluster effects in lectin binding and might represent useful tools for MR targeting and imaging.

Recently, an interesting gene delivery approach based on MR targeting was reported [97]. The system consists of a cyclodextrin platform containing a polycationic cluster and a multi-tail hydrophobic moiety. In the presence of plasmid DNA, nanometric complexes are formed that are suitable for gene transfection (Fig. 3D). Nanoparticles formed by self-assembly of multivalent mannosylated polycationic cyclodextrins and pDNA were recognized by the MR and used for transfection of macrophages in vitro. In another glycotargeting approach, glycocluster conjugates with a $CD8^+$ epitope of the Melan-A/Mart-1 melanoma antigen were synthesized [98]. While fluorescent-labeled Melan-A glycoclusters containing either a dimannoside or Lewis oligosaccharide were internalized by DCs, no uptake was observed for glycocluster conjugates containing lactose. An efficient Melan-A-specific $CD8^+$ T cell response was induced rendering synthetic glycocluster-tumor antigen conjugates promising tools for tumor vaccines or immunotherapy.

Targeting of DCs by particulate systems such as polyelectrolyte capsules was reported as an efficient system for antigen delivery [99]. Particles can further be glyco-modified to increase targeting specificity. For this purpose, natural polysaccharides as well as synthetic oligosaccharides were used. In one study, isolated glucomannan modified with *N*, *N*′-carbonyldiimidazole/ethylenediamine served as a gene carrier [100]. Cationized glucomannan formed complexes with oligodeoxynucleotides thus mediating transfection. A high targeting specificity for macrophages was observed suggesting MR- and/or Dectin-1-mediated endocytosis. To optimize targeting of cellular compartments and to provide for a triggered release of the antigens, pH-sensitive particles were synthesized displaying mannose-based ligands at the surface [101]. Formulation of vaccine antigens to be released by either pH shift or enzymatic cleavage in endosomal compartments of the cell may thus be a means to further increase targeting specificity and triggered release. As an alternative carrier system, mannosylated chitosan/DNA nanoparticles were used [102]. This system was successfully employed for IL-12 gene delivery into DCs. Mannose-bearing chitosan microspheres containing complexes of hepatitis B virus DNA and PEI were also used [103]. These microspheres allowed for MR-specific targeting and an efficient release of the DNA/PEI complex. Polyanhydride nanoparticles functionalized by covalent linkage of dimannose were employed for targeting CLRs on APCs [104]. Incubation of bone marrow-derived DCs with the carbohydrate-functionalized particles led to an increased expression of MHC class-II molecules as well as co-stimulatory molecules. To investigate multivalent effects in the binding of carbohydrate ligands with their respective lectin receptors more in detail, a spatially defined presentation of glyco-ligands is needed. Solid-phase polymer synthesis enables access to sequence-defined monodisperse glycopolymer segments as tools for lectin receptor-specific targeting [105].

In numerous studies, glycoliposomes were successfully used for targeted delivery into APCs as well as stimulation of immune responses. OVA-encoding DNA encased by mannosylated cationic liposomes was used for transfection of DCs leading to higher OVA expression levels compared to unmodified liposomes in vitro. Administration of the mannosylated liposomes in vivo led to higher OVA mRNA levels in spleen and the peritoneal cavity [106]. In addition to cell-specific targeting, mannosylation is capable of shaping an initiated immune response. In a DNA-based HIV vaccination model, the use of mannan-coated liposomes led to an increased HIV-specific DTH response [107]. In addition, the mannan-coated liposomes enhanced cytotoxic T cell activity in an IFN-γ-dependent manner. This finding indicates that mannan liposomes can be used to direct vaccination-induced immune responses towards Th1. To investigate whether alveolar macrophages could be specifically targeted by mannosylated liposomes, ciprofloxacin was incorporated in liposomes. Upon pulmonary administration, mannosylation led to increased antibacterial effects compared to unmodified liposomes loaded with ciprofloxacin [108]. In another study, the endocytosis of mannosylated liposomes in primary cultured alveolar macrophages and the in vivo uptake upon intratracheal administration in rats were analyzed [109]. The in vitro uptake of Man-liposomes occurred in a concentration-dependent manner and was inhibited by an excess of mannan indicating MR-mediated endocytosis. Again, efficient targeting of the intratracheally applied mannosylated liposomes to alveolar macrophages was observed. These studies indicate that organ-specific targeting of APCs using mannosylated liposomes is possible.

Immunization of mice with OVA-loaded oligomannose-coated liposomes and challenge with OVA-expressing E.G7-OVA tumor cells led to complete tumor rejection [110]. Even in established tumors, therapy with OVA encased in oligomannose-coated liposomes significantly reduced tumor growth indicating the potent adjuvant activity of liposome mannosylation. In another study, mannosylated cationic liposomes were used for targeted delivery of melanoma-associated antigen expressing DNA into macrophages and DCs for inducing anti-cancer immune responses [111]. Mannosylated cationic liposomes led to higher gene transfection rates than unmodified liposomes, elicited pronounced $CD8^+$ T cell responses against melanoma cells and inhibited tumor growth. Moreover, mannosylated liposomes were used for encapsulation of magnetic nanoparticles for hyperthermia therapy in a murine cancer model. Loading of oligomannose-coated liposomes with 5-fluorouracil and injection into the peritoneal cavity led to accumulation in the omentum and other lymphoid tissues [112]. When mice were co-administered with 5-fluorouracil and magnetic nanoparticles encased in oligomannose-coated liposomes followed by treatment with an alternating magnetic field, tumor growth could be controlled. In an OVA tumor model, immunization of mice with oligomannose-coated liposomes loaded with OVA induced marked OVA-specific $CD8^+$ T cell responses indicating efficient cross-presentation [113]. An interesting strategy is the use of mannosylated niosomes as a delivery platform and adjuvant for oral vaccine delivery [114]. Niosomes were loaded with the antigen tetanus toxoid (TT) and were coated with a modified polysaccharide o-palmitoyl mannan. Oral administration of these liposomal vaccine carriers resulted in increased antigen-specific IgG responses compared to alum-adsorbed TT or uncoated liposomes. In addition, significant levels of sIgA were detected in mucosal secretions indicating a marked mucosal immune response.

Besides their use as adjuvants for prophylactic or therapeutic cancer vaccination, oligomannose-coated liposomes were also used to induce potent immune responses against pathogens rendering them promising targeting platforms for vaccines against infectious diseases. When soluble leishmanial antigen was entrapped into oligomannose-loaded liposomes and mice were immunized with these constructs, strong Th1 immune responses were induced accompanied by marked levels of IFN-γ and IL-2 [115]. Immunized mice were efficiently protected upon challenge with *Leishmania major* indicating that the induced Th1 immune response was *Leishmania*-specific and protective. Similar results were obtained in a model of neosporosis infection [116]. Immunization of mice with *Neospora caninum* dense granule protein 7 entrapped in mannotriose-coated liposomes led to parasite specific Th1 responses and induced humoral responses in vaccinated mice.

A comparison between mono-, di-, and tetraantennary mannosyl lipid derivatives revealed that liposomes prepared with multibranched mannosylated lipids displayed higher binding affinity for the MR compared to monomannosylated analogs [117] (Fig. 3E). Interestingly, diantennary mannosyl lipids were as efficient as the tetraantennary lipid which might indicate that the diantennary ligand is efficiently

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engaged in multivalent interactions. An important question for efficient CLR targeting is how ligand density on the surface of liposomes or other carrier systems might influence receptor-mediated endocytosis. Indeed, in a study on MR targeting using mannosylated emulsions, the mannose density crucially influenced cellular recognition and internalization [118]. Recently, surface-modified stealth microspheres were prepared by electrostatic surface assembly of mannan onto previously formed adlayers of poly(L-lysine) or by assembly of poly(L-lysine)-PEG capped with mannoside ligands [119]. Indeed, ligand density significantly influenced the endocytosis of the mannoside-loaded microspheres. This study indicates that a systematic investigation is important to determine the influence of the ligand density on targeting efficacy.

To date, most studies have focused on carrier systems modified by mannose residues. Very promising results have been observed using mannosylated proteins such as tumor antigens and vaccine antigens that were able to induce substantially increased immune responses compared to unmodified antigens. Numerous studies also demonstrated the utility of mannosylated liposomes in vitro as well as in murine infection and tumor models. These findings indicate that mannosylated carrier systems are suitable for APC targeting in order to shape immune responses. However, often discrimination between specific MR targeting and the involvement of other CLRs such as DC-SIGN is difficult. Thus, a current challenge is to further optimize MR ligands either by synthesis of defined high-mannose glycan structures or by designing high-affinity MR ligands to enhance the specificity of MR targeting.

In conclusion, glycan-based targeting systems such as liposomes, dendrimers, polymers, nanoparticles, and carbohydrate-modified proteins were successfully employed for DC and macrophage targeting. Carbohydrate-based targeting approaches may also be useful for modulating cell growth, cell migration or other effector functions such as cytokine release as demonstrated recently using carbohydrate-functionalized surfaces [120].

3.3. Other C-type lectin receptors

Other CLRs in innate immunity were also exploited for cell-specific targeting using multivalent ligand display. One example is the macrophage galactose-type lectin (MGL) which is closely related to the liver-specific ASGPR but is expressed by immature DCs and macrophages [121]. Determination of the carbohydrate binding pattern of human MGL revealed specificity for terminal GalNAc structures that are present on the mucin MUC1 as well as CD45 on T effector cells [122]. Enzymatic glycosylation of MUC1 with GalNAc moieties was efficient in inducing internalization by monocyte-derived DCs mediated by the MGL receptor [123]. In another study, the preferred recognition of the tumor antigen Tn by MGL was exploited for specific targeting of mouse and human DCs [124]. Immunization with Tn-modified glycopeptides led to antigen-specific T cell priming and B cell responses indicating the potential of in vivo DC targeting based on Tn–MGL interactions. Targeting of MGL on immature DCs by a Tn-carrying MUC1-glycopeptide triggered phosphorylation of the MAP kinases ERK1 and 2 and led to NFκB activation [125]. In addition, MGL targeting induced DC maturation and provoked a tumor antigen-specific $CD8^+$ T cell response. In the murine system, specific targeting of the MGL isoform MGL2 was shown by GalNAc modification of antigens again resulting in efficient $CD4^+$ T cell responses and enhanced cross-presentation [126]. Defined presentation of carbohydrate ligands of the murine MGL1 and MGL2 isoforms on a DNA backbone also demonstrated the suitability of MGL targeting [127].

Other CLRs that were exploited for cell specific drug/gene or antigen delivery by carbohydrate ligands include the DC/macrophage-expressed Dectin-1 that was targeted by the β-1,3-glucans schizophyllan [128] and laminarin [129], the selectins [130], and CD69, an early activation marker expressed by activated T cells and natural killer (NK) cells [131,132]. Table 1 shows examples and summarizes current strategies used for targeting CLRs in innate immunity.

4. Conclusions

Targeting CLRs is an attractive strategy not only for drug/gene delivery as shown for ASGPR in the liver, but also for DC/macrophage targeting to modulate immune responses. Multivalent display of CLR ligands such as carbohydrates or glycomimetics has become a powerful method. To gain access to complex carbohydrates used for targeting, novel synthetic strategies such as chemoenzymatic methods and automated carbohydrate synthesis have been developed and further optimized in the last years [133]. This progress is accompanied by major advances in the development of array platforms [134]. Glycan arrays have helped identify novel glycan ligands of CLRs that may be used for CLR-specific targeting and the modulation of immune responses in vivo [135,136]. To target CLRs specifically using multivalent ligand display, it is necessary to obtain structural information about the respective CLR and its binding to carbohydrate ligands. In this regard, glycomics and NMR-based techniques as well

Table 1
Examples of current strategies for antibody-mediated and carbohydrate-based targeting of CLRs in innate immunity.

Antibody-mediated CLR targeting	DEC-205 targeting to induce tolerance or immunity		[10,11]
	DC-SIGN/MR targeting to enhance immune responses		[46,139]
	DC immunoreceptor (DCIR) targeting to induce cross-presentation		[12]
	Clec-9a targeting for tumor therapy/targeted vaccine delivery		[13,14]
	Targeting of other CLRs (e.g. DCAR, Dectin-1, Dectin-2, Clec-12a) to enhance immune responses		[140–143]
Multivalent ligand display for CLR targeting	Glycan-modified antigens	DC-SIGN targeting with glycan-modified antigens to enhance antigen presentation	[48]
		MR targeting with mannosylated antigens to increase anti-tumor responses or cross-presentation	[79,81,84,91]
		GalNAc modification of antigens for MGL targeting on DCs	[123–125]
	Glyconanoparticles	Multivalent display of HIV gp120 high-mannose oligosaccharides	[51,52]
		Mannosylated chitosan/DNA particles for gene delivery into DCs	[102,103]
		Mannosylated polyanhydride nanoparticles for targeting APCs	[104]
	Glycodendrimers	Mannosylated dendrimers to inhibit DC-SIGN binding to HIV gp120	[53,54]
		DC-SIGN targeting by glyco-dendri-protein-nanoparticles to inhibit Ebola virus infection of cells	[58]
		Mannosylated PAMAM dendrimers for antigen delivery into APCs	[93]
	Glycofullerenes	DC-SIGN targeting to inhibit Ebola virus infection of cells	[59]
	Glycoliposomes	Glycan-modified liposomes for DC-SIGN-based antigen delivery	[60,61]
		APC targeting with mannosylated liposomes to increase antigen presentation/anti-tumor responses	[108–110,113]

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as X-ray structure analysis have become indispensable tools to provide a deeper insight into CLR/glycoconjugate interactions [137]. A number of carrier systems have been employed to display carbohydrates in a multivalent fashion, including nanoparticles, dendrimers, polymers, and liposomes [138]. Advances in carrier system design will provide further insight into the optimal ligand display and how ligand density influences targeting and signaling pathways. This will lead to even more specific CLR targeting in the future for targeted vaccine delivery, tolerance induction, and tumor therapy.

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