Targeting of antigen presenting cells with mannosylated conjugates

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Chapter 1

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Introduction

The immune system consists of a large variety of cells that continuously control and protect the body against foreign and aberrant cells. It can be divided into an innate and an adaptive part, that recognize these malignant cells through non-specific general traits (the innate part) or through highly specific interactions, as developed in the adaptive part of the immune system. Innate immune cells can instantly battle pathogens and aberrant cells after sensing danger or pathogen-associated molecular patterns (DAMPs and PAMPs). When the innate system does not suffice in eradication, adaptive immune cells are recruited to generate a selective response. The adaptive immune system serves two goals. It generates a strong tailored immune response with high specificity, and secondly, it generates lasting immunity by the formation of memory cells. Upon recognition of their specific target, the adaptive immune cells start to proliferate to form large numbers of specific cells. A small portion of these cells transforms into memory cells, which can be readily reactivated and allows the immune system to generate a fast and specific response when re-challenged by the same pathogen or aberrant cell.

The adaptive immune system has been exploited for centuries in the treatment of diseases. At the end of the eighteenth century, Edward Jenner successfully immunized the first human by challenging his immune system with cowpox, thereby effectively protecting him from smallpox.[1] Since Jenner, many other therapies that exploit the power and specificity of immune cells have been developed. In the last decades, immunotherapies have revolutionized cancer treatment with the development of chimeric antigen receptor (CAR) T cells and checkpoint inhibitors.[2,3] The inhibition of checkpoints can result in the restoration of immune responses and has successfully treated various tumors were traditional cytotoxic therapies failed.[4,5] Immunotherapies often rely on T cells, a specific set of adaptive immune cells and the amount of tumor-infiltrating T cells has been shown to be a prognostic marker for success in immunotherapy.[6,7] However, the presence of T cells does not guarantee a sufficient response if the T cells are not tumor-reactive. For example, by sequencing the T cell receptor (TCR) of intratumoral T cells, Scheper et al.[8], demonstrated that the majority of the T cells were not tumor-reactive. Furthermore, other immune cells, besides T cells, are required to generate a long-lasting response against malignant cells.[9] A possible method to improve T cell based therapies is by active immunization against cancer cells by challenging the immune system with specific tumor-associated antigens (TAAs) to mount a specific T cell response to target aberrant cells or help improve and elongate the immune-response.[10,11]

Innate immune cells can distinguish foreign and damaged cells from normal cells using pathogen recognizing receptors (PRRs). These receptors recognize distinct molecular motives that have been preserved in pathogens such as viral and bacterial DNA, RNA, carbohydrates, and lipids. Upon recognition, the innate immune cells are activated and generate signals to recruit other immune cells to the site of infection. Among the innate cells are dendritic cells (DCs) that play a pivotal role in the activation of the adaptive immune system. DCs are antigen-presenting cells (APCs) that can present (peptide) antigens on their cell surface in a protein called the major histocompatibility complex (MHC). Two types of (classic) MHC proteins exist of which class I (MHC-I) presents antigens from endogenous proteins from the cytosol and class II (MHC-II) antigens from endocytosed (pathogenic) proteins. MHC-I is present on all cells and presents epitopes containing 8-11 amino acids. It allows for the detection of aberrant cells through the interaction with cytotoxic T cells (CTL or $CD8^+$), to induce programmed cell death. MHC-II is only expressed by professional APCs such as dendritic cells (DCs), B cells, and macrophages. MHC-II presents epitopes with a less stringent size restriction (generally in the 13-17 amino acids length range) to T helper cells (T_h cells or $CD4^+$), that in turn stimulate effector cells and help prolong the immune response.[12] T cells recognize the combination of MHC occupied with an epitope via the T cell receptor (TCR). Although antigens presented in MHC-II are obtained *via* endocytosis of pathogens

and antigens presented in MHC-I are derived from the cytosol, professional APCs present a small amount of the endocytosed antigen in the MHC-I. This route is called antigen cross-presentation and allows for immunity against tumors and viruses.[13] Importantly, the recognition of epitope-MHC is not sufficient to activate T cells and additional stimuli from the APC, in the form of co-stimulatory proteins such as CD40 and cytokines, are required. APCs upregulate the levels of these stimuli when their PRRs recognize PAMPs.

Mammalian immune cells express a multitude of PRRs which are divided into subfamilies based on their structure and the ligands they bind. The four well-defined families are the C-type lectin receptors (CLRs), toll-like receptors (TLRs), nucleotide-binding oligomerization domain-like receptors (NOD-like or NLRs) and the retinoic acid-inducible gene-I-like receptors (RIG-like, or RLRs).[14] Occasionally, new PRRs are identified that could potentially increase the number of PRR families.[15] The focus of this thesis is on the CLR and TLR families. TLRs recognize different type of PAMPs such as bacterial lipopeptides and bacterial and viral RNA and DNA and have been extensively explored to acquire adjuvants for vaccine development.[16,17] CLRs recognize viral, bacterial, and fungal derived glycans. Both soluble and transmembrane CLRs exist that bind carbohydrates in a calcium depending manner. The transmembrane CLRs are classified into two classes based on whether the position of their N-terminus is extra- (type I) or intra-cellular (type II). The CLR family recognizes various carbohydrates, for example, dectin-1 recognizes β-glucans, and the macrophage galactose-type lectin (MGL) recognizes *N*-acetyl galactosamine containing structures. Several CLRs can recognize mannose structures, which is the main subject of the research described in this Thesis. These include the mannose-binding lectin (MBL), the mannose receptor (MR, or CD206), dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN or CD209), and Langerin (CD207) and are discussed in the following sections.

MBL

The mannose-binding lectin (MBL) is a soluble CLR, which contains an N-terminal cysteine-rich region and a C-type lectin domain that can bind mannose, fucose, and GlcNAc type of carbohydrates. The cysteine-rich domain forms disulfide bonds with other MBL peptides, creating a trimeric structure with 45 Å spacing between the CRDs.[18] These subunits can multimerize further into a tetrameric complex, forming a bouquet-like structure with 12 CRDs per complex (See Figure 1).[19] The affinity of a single MBL protein is low, but when multimerized it can bind with high avidity to the neutral carbohydrates mentioned above.[20] Pathogen recognition by MBL can initiate activation of the innate complement system via the lectin pathway.[21] Additionally, binding of MBL enhances phagocytosis,[22] thereby trafficking pathogens towards phagosomes, where it can lead to

the engagement of TLR2/TLR6, and MBL can thus act as a TLR co-receptor.[23] Due to the complexity of the complement system, antigen targeting using MBL is hardly explored. However, it can help to target antigens toward germinal centers which could start an appropriate adaptive immune response.[24]

MR

The mannose receptor (MR, or CD206) is a C-type lectin receptor that is found on the surface of endothelial cells, macrophages, Langerhans cells (LCs) and (immature) DCs. The MR occurs both as a monomer and dimer, and both complexes can bind mannosides,[25,26] but dimerization is required for the binding of larger particles such as HIV-1.[27] The receptor consists of a short C-terminal intracellular domain (type I CLR), a transmembrane domain linked to eight C-type carbohydrate recognition domains (CRDs), which can bind mannose, fucose, and *N*-acetyl glucosamine containing carbohydrates in a Ca^{2+} dependent manner.[26,28] These are followed by a fibronectin type-II domain and a cysteine-rich domain on the N-terminus (see Figure 1). The cysteine-rich domain can bind sulfated carbohydrates in a Ca^{2+} independent manner,[29,30] and the fibronectin domain can bind and endocytose collagen.[31] Human MR has eight CRDs with only a small amount of homology between them and varying affinities towards mannose structures.[32] CRD-8 is the closest to the C-terminus and the transmembrane domain. Of all eight CRDs, only isolated CRD-4 is able to bind mannosides with a significant affinity, and it binds monosaccharides with similar specificity as the MBL.[33] However, CRDs 4-8 are required to achieve the binding affinities of the natural MR, indicating that these also have a role to play in the binding of mannosides.[25,28] Targeting antigens towards the MR can serve two functions: enhancing cell maturation and antigen presentation. Although the MR lacks an intracellular signaling motive, engagement of the receptor can induce cytokine production, although the pathway through which this occurs remains unknown[29,34] and it has been speculated that other mannose-binding receptors are responsible for these signals.[35] Colocalization of the MR and antigen suggest that they can be transported together toward early endosomes, which enables the cross-presentation of the antigen.[36,37] Together, these findings make the MR an attractive target for cell-specific vaccine development, as described in previous reviews.[38,39] However, it has proven to be challenging to study the binding affinity of this receptor *in vivo* because of the low expression levels of the MR on the cell surface. In addition, the poor stability of the receptor, when overexpressed, complicates the determination of ligand affinity *in vitro*. The stability of the MR can be improved through small alterations in the receptor's amino acid sequence, but this often modifies the properties of the receptor.[40]

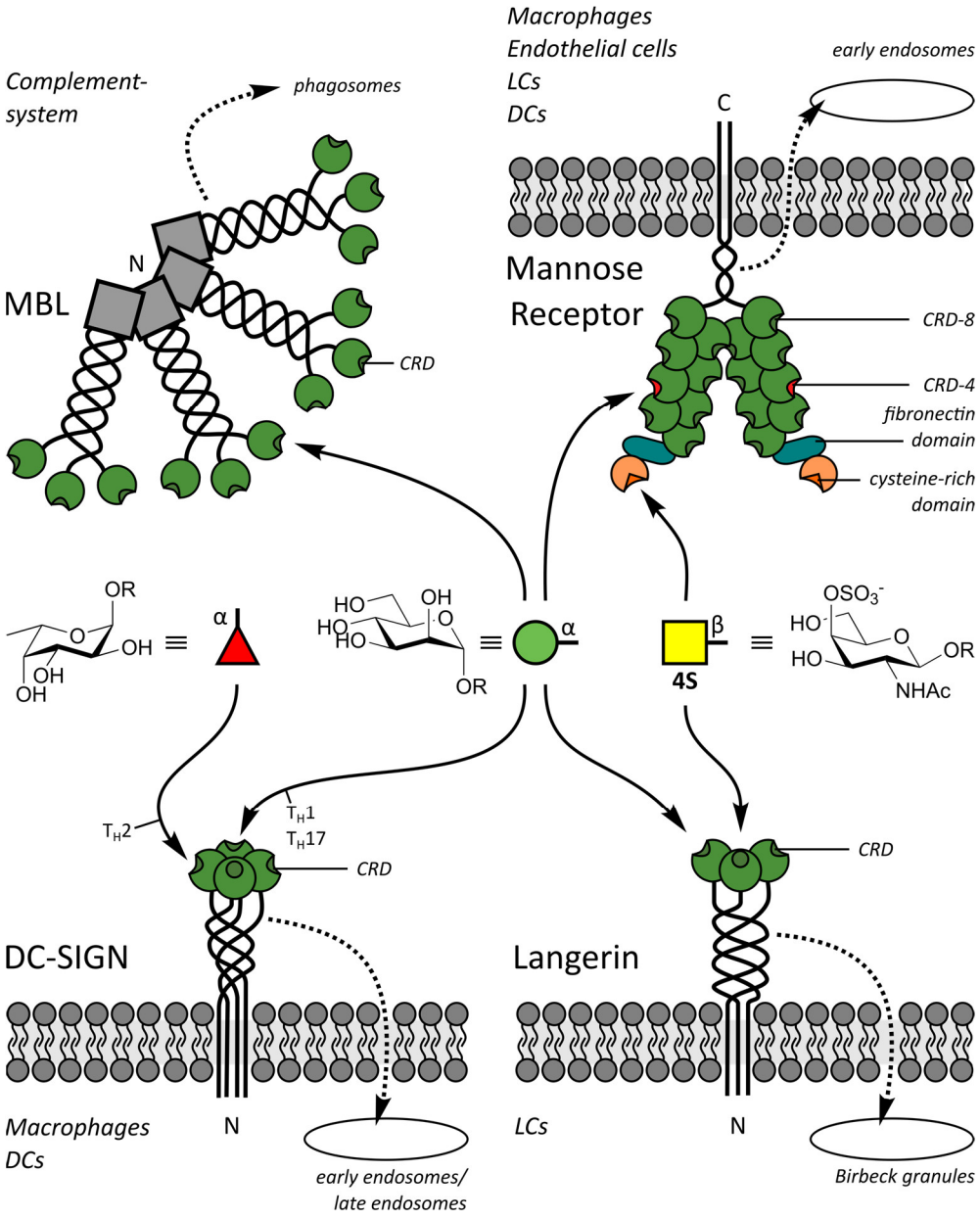
DC-SIGN

Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN or CD209) is a C-type lectin present on dendritic cells and specific macrophage subsets.[41] DC-SIGN is a type II CLR and bears multiple internalization motives and a signaling motive on its intracellular domain. The extracellular part of DC-SIGN contains a flexible neck region and a CRD that can both bind mannose, fucose, and GlcNAc-like structures. DC-SIGN multimerizes into tetrameric structures on the cell surface, improving the binding avidity to pathogens (see Figure 1).[42] In these tetrameric structures, the minimal distance between CRDs is 40 Å.[43] Upon binding of these CRDs, the receptor can induce signaling and it has been shown that mannosylated antigens can activate Ras-1 signaling resulting in an inflammatory response.[44] Binding the same CRD with fucosylated antigen, however, induces a different inflammatory response resulting in different T cell subsets. Thus, DC-SIGN can effectively skew the T cell response to stimulate T_H1 or T_H17 cell (through mannoside activation) or induce a T_H2 response (by binding of fucoses).[44–46] DC-SIGN is also a scavenging receptor that can rapidly internalize antigens upon binding.[47–49] These combined functions make DC-SIGN an attractive target for vaccine development.[50,51] DC-SIGN mediated endocytosis can traffic antigens towards different types of endosomes.[52] For example, large structures such as HIV-1 are trafficked towards late endosomes/lysosomes resulting in MHC-II presentation,[53] while smaller fragments can be trafficked towards early endosomes, thereby improving cross-presentation.[54]

Langerin

The skin is an attractive site for vaccinations since it contains large quantities of Langerhans cells (LCs), professional APCs which are a subset of DCs.[55] LCs express the CLR Langerin (CD207),[56] which is a type II transmembrane protein with a CRD that has a preference for mannose, fucose, and GlcNAc, similar to DC-SIGN. Affinity studies with an array of carbohydrates suggest that langerin can also bind sulfated oligosaccharides.[57,58] The receptor is expressed as a trimeric complex on the cell surface, binding multivalent carbohydrates (see Figure 1). Unlike DC-SIGN, scavenging by Langerin traffics antigens to Birbeck granules instead of endosomes. These Birbeck granules can degrade particles such as viruses, which allows LCs to act as a natural barrier against viral infections, for example, by HIV-1.[59,60] These findings have sparked a large interest in the development of ligands that are either specific for Langerin or DC-SIGN.[58,61] More information on vaccine strategies via Langerin is reviewed by Dam et al.[51] and Stoitzner et al.[62]

Figure 1: Mannose-binding C-type Lectin Receptors.



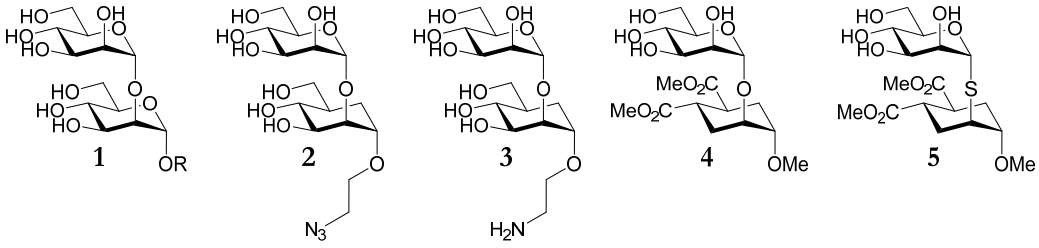
Schematic representation of mannose-binding C-type lectin receptors and the ligands that bind the different binding domains of the receptors.

Targeting of mannose-binding CLRs

Endocytosis by APCs via CLRs has gained much attention in the last decades, focusing on two distinct approaches. Ligands have been developed to block CLR-mediated endocytosis to prevent pathogens from hijacking the CLR internalization pathway for transfection. Alternatively, CLR-binders have been developed to target specific cargo into APCs *via* the CLRs. Although both strategies serve opposite functions, the design principles underlying the development of ligands

designed for either purpose is of relevance for the other application as well. The design of endocytosis blocking ligands has resulted in a large number of glycomimics that can bind with high affinity and selectivity for one CLR over another.[63–69] Besides improved affinity and selectivity, replacing native ligands with glycomimics can improve their stability against enzymatic degradation.[70] For example, Bordoni et al.[71] synthesized **2** and **3**, carba-analogues of the α1,2-dimannoside **1**, which lack the endocyclic oxygen, as enzymatic stable ligands for DC-SIGN (Figure 2). Tamburrini et al.[72] improved the stability of the α1,2-glycosidic bond in carba pseudo mannoside **4** by the introduction of a thioglycosidic bond, forming pseudo disaccharide **5** (Figure 2). The affinity for CLRs can be further enhanced by the multivalent presentation of mannoside(-mimic)s by clustering the ligands on different types of carriers. Viral infection can be stopped effectively using ligands with nM affinities based on carriers systems such as dendrimers,[73,74] molecular rods,[75] gold nanoparticles (AuNPs),[76,77] and polymers.[43]

Figure 2: Stabilized pseudo mannosides.



Exploiting CLRs to target antigens towards antigen-presenting cells

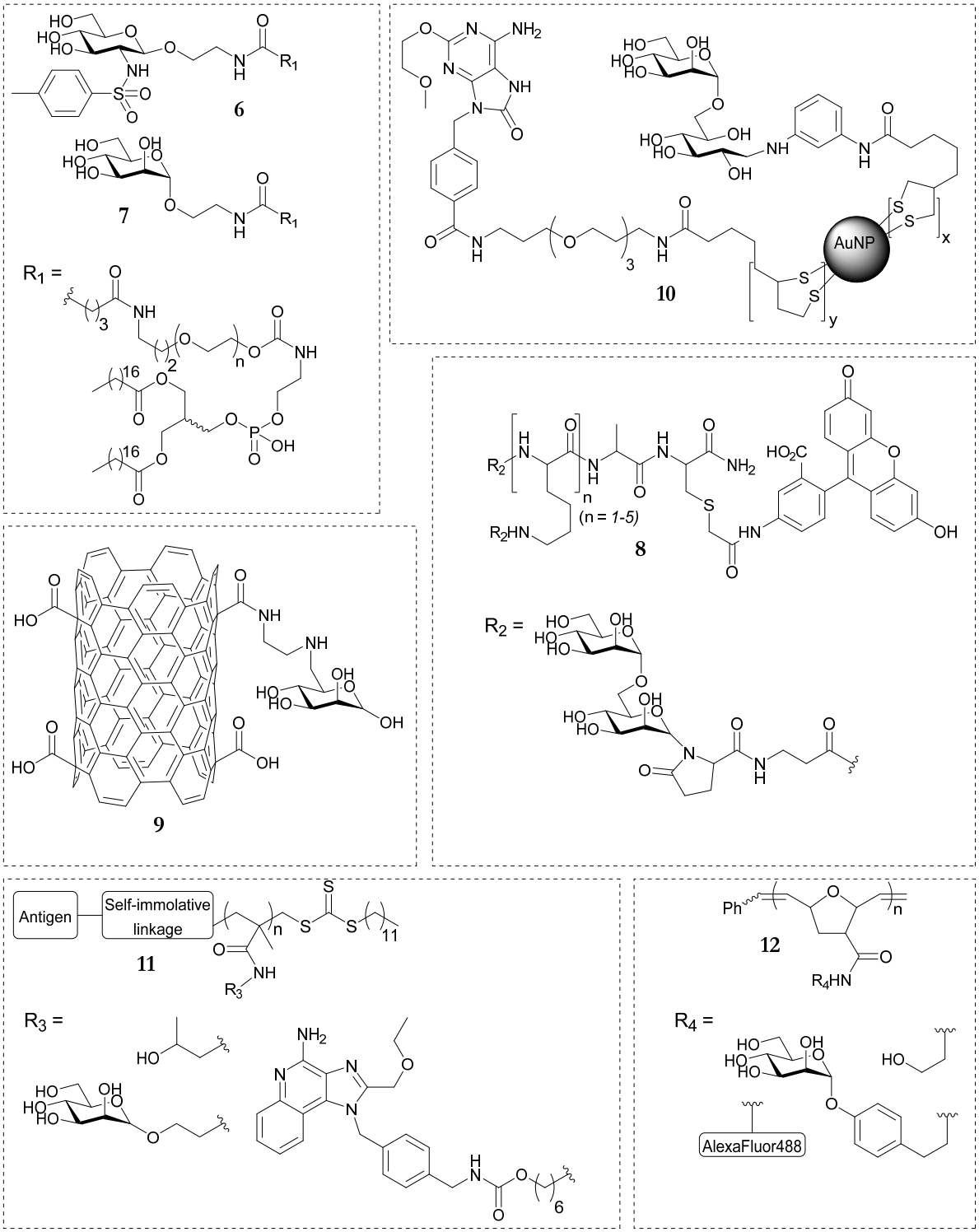
The second approach to utilize CLR mediated endocytosis aims at the delivery of cargo towards APCs. One potential method to achieve this comprises the use of antibodies. Sehgal et al.[78] have recently reviewed different strategies for DC vaccination, including anti-CLR antibody conjugates. Cruz et al.[79] combined antigen-coated nanoparticles with anti-DC-SIGN antibodies and Breman et al.[80] combined anti-MR antibodies with a peptide antigen to deliver the antigens to APCs. Although both approaches improved the uptake efficacy, the level of T cell reactivity was similar to unconjugated antigens, suggesting that antigen presentation was not improved.

Carriers to deliver antigens.

Targeting CLRs with mannosylated constructs is a popular method to deliver cargo to APCs. This targeting is often achieved with multivalent carriers that contain mannosides and a cargo of interest, such as an antigen, and this approach has been reviewed extensively.[78,81,82] Carriers exploited for selective targeting are often similar to those used for blocking of viral entry. For example, Zhu et al.[83] reported a mannosylated cationic lipid-hybrid polymersome, that combined

an ovalbumin antigen with two TLR agonists (Imiquimod and monophosphoryl lipid A) and induced strong activation and a synergistic antitumor immune response. Schulze and Wamhoff et al.[61,84] selectively targeted antigens to LCs, using liposomes coated with Langerin specific glycomimic **6** or mannoses **7** (Figure 3). Liposomes with **6** could selectively deliver their content (e.g., fluorophore[84] or Doxorubicin[61]) to Langerin$^+$ cells when compared with the mannosylated liposomes which were also endocytosed by other CLRs. As a proof of concept, Frison et al.[85] used an oligopeptide carrier with lysine repeats that were functionalized with carbohydrates to provide constructs such as **8** (See Figure 3). Incorporation of a fluorescein label allowed to track the uptake and routing of the conjugates via either the MR or DC-SIGN. Their results have shown that binding avidity increased with a higher number of mannosides (n=2 < n=3) and also that fucosylated constructs (Lewis A, Lewis B, or Lewis X) could be internalized by DC-SIGN, but not by the MR, demonstrating that these receptors can be discriminated using the appropriate glycans. Dong et al.[86] grafted mannosides on carbon nanotubes (**9**) which could adsorb a model OVA antigen. These nanotubes were efficiently engulfed by DCs indicating that such nanotubes could be potent nanovectors for antigen delivery, which could lead to selective drug delivery applications. Shinchi et al.[87] conjugated both mannosides and a TLR7 agonist to gold nanoparticles (**10**, Figure 3), which improved the activity of the TLR7 ligand. Co-administration of these nanoparticles with OVA as a model epitope resulted in a more efficient presentation due to improved activation of the APC. Wilson et al.[88] developed methacrylic acid co-polymers equipped with mannosides and a resiquimod analog as side groups, that were reversibly conjugated to an antigen (**11**, Figure 3). When both the mannoside and resiquimod were combined in a single polymer, the humoral response and the cellular immunity were improved. These results demonstrate that the introduction of ligands for both TLR7 and mannose-binding CLRs in one construct can improve the effectiveness of the immune response. Another mannosylated polymer carrier was synthesized by Jarvis et al.,[54] who utilized a ring-opening polymerization approach to generate multiple functionalized polymers (**12**, Figure 3). Both soluble polymers and polymer aggregates were obtained, and the fate of antigen routing proved to be dependent on the physical properties of the carrier.[89] These results showed that soluble antigen is routed toward early endosomes, ideal for antigen cross-presentation,[52] while aggregates are directed to compartments that are more suitable for CD4$^+$ presentation.[90] This size-dependent routing is not only affected by the size of the carrier, but also by the type of CLR targeted. When Fehres et al.[91] compared Lewis Y functionalized synthetic long peptides (SLPs) with liposomes, the routing fate and antigen presentation capacity proved to depend on the CLR responsible for the trafficking. The best antigen presentation *via* langerin was achieved with SLPs, and presentation *via* DC-SIGN using antigen-loaded liposomes.

Figure 3: Multivalent CLR targeting carriers.



Although multivalent systems, such as those described above, have been successfully applied for improvement of antigen (cross-)presentation, the heterogeneous character of the carriers can result in incoherent effects. Therefore, much effort has been directed at the development of well-defined

single molecules such as synthetic long peptides and defined dendrimers. For example, based on the results obtained with **8**, Srinivas et al.[92] synthesized constructs such as **13** (Figure 4) in which four repeating lysines were functionalized with glycosyl residues and conjugated to a Melan-A/Mart-1 melanoma epitope (Melan-A_{16-40}). The antigen cross-presentation was enhanced by binding to MR or DC-SIGN. Similar immunological results were obtained by Rauen et al.[93] who generated mannosylated SLPs (**14**, Figure 4) comprising a lysine residue with two α-mannosides connected to either the MHC-I restricted $OVA_{257-264}$, the MHC-II restricted $OVA_{323-339}$, or the MHC-I restricted HPV $E7_{43-63}$ epitope. It was demonstrated that mannosylation[94,95] of the synthetic long peptide enhanced cross-presentation but not MHC-II antigen presentation, indicating that the mannosides in this construct routes the antigen towards the early endosomes. Grandjeun et al.[96] developed a synthetic approach to generate mannosylated dendrimers (**15**, Figure 4) to specifically target mannose-binding CLRs on DCs. Their dendrimers are based on branching lysines that were conjugated to an epitope via an N-terminal hydrazino-ligation. In an alternative approach, McIntosh et al.[97] conjugated one or two complex Man_9 structures to a synthetic peptide using an enzymatic glycosylation strategy to form native N glycan **16**. The mannosylation improved binding to APCs, and the antigen was effectively presented as long as the epitope was not glycosylated. Glaffig et al.[98] combined a MUC-1 epitope with both a mannose targeting moiety and a tetanus toxoid (TTox) as an helper T cell epitope *via* squarate conjugation (**17**, Figure 4). Mouse immunized with this construct exhibited stronger IgG antibody titers in comparison with a control construct that lacked the mannosides.

The incorporation of additional adjuvants can further improve the effectiveness of mannosylated antigens.[99] For example, Moyle et al.[100] synthesized mannosylated conjugates **18** bearing an HPV $E7_{44-62}$ epitope and a lipid-core-peptide (LCP) adjuvant.[101] The trifunctional conjugates were able to protect against TC-1 tumor cells. Sedaghat et al.[102] synthesized similar constructs in which an $OVA_{323-339}$ MHC-II epitope was combined with self-adjuvating lipids, a reporter group, and targeting mannosides (**19**, Figure 5)**.** Both the lipids and mannosides in the constructs played a significant role in the receptor-mediated uptake.

Figure 4: Mannosylated antigen.

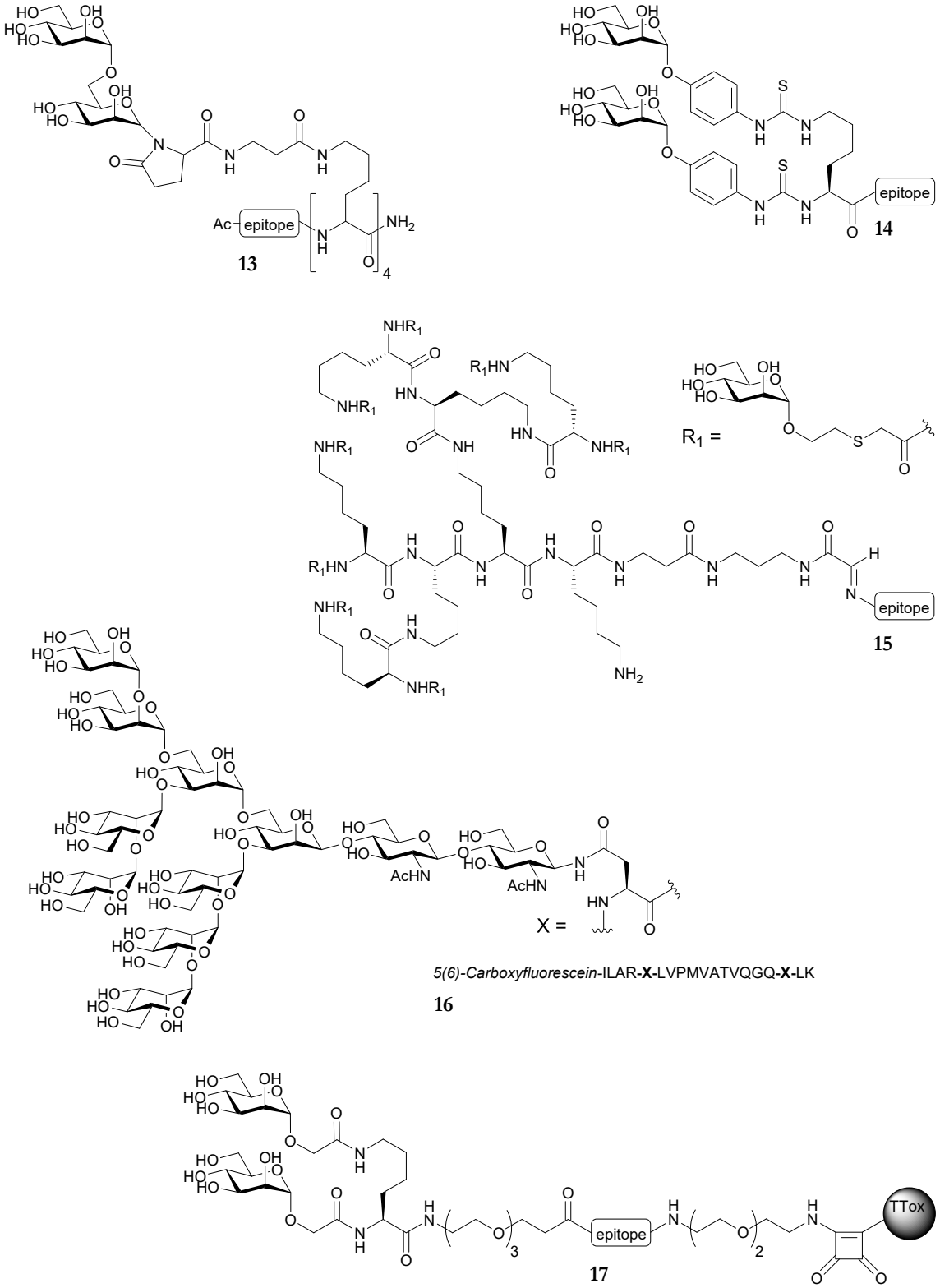
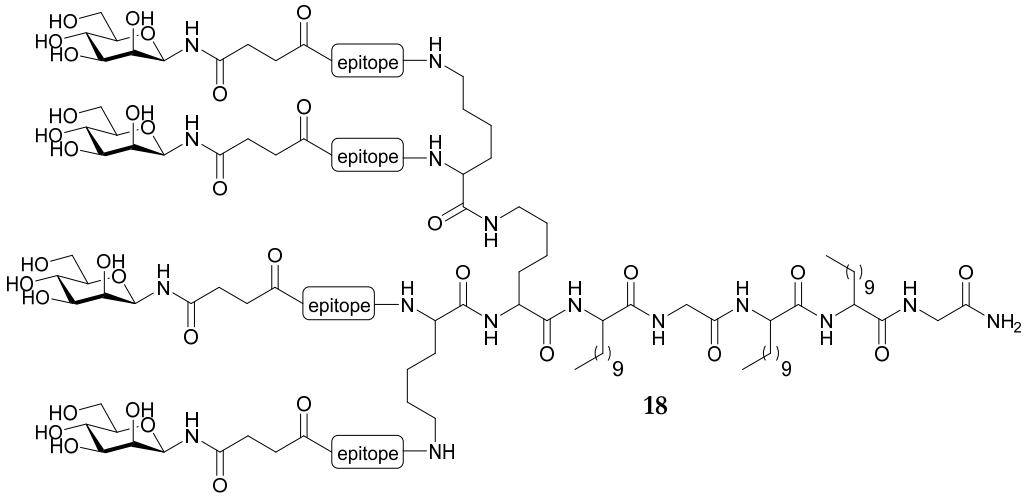
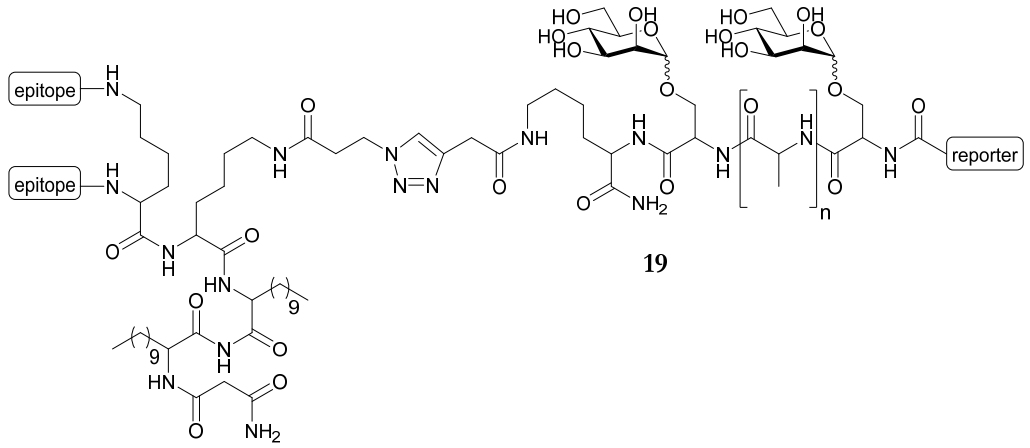


Figure 5: Mannosylated trifunctional conjugates.





Outline of this Thesis

This Thesis presents studies on the targeting of mannosylated conjugates to C-type lectin receptors (CLRs) present on antigen presenting cells. **Chapter 2** describes a systematic approach to determine the effect of both the number and type of mannosides on the affinity for the three mannoside binding transmembrane CLRs: the MR, DC-SIGN, and Langerin. The affinities of the clusters was determined using different *in vitro* techniques, including a new method that utilizes super-resolution microscopy. The established affinities directed the selection of the mannoside clusters to be used in follow-up studies in this Thesis. **Chapter 3** describes improvements in the synthesis of a known Toll-like receptor (TLR) agonist which allows the use in solid-phase peptide synthesis. This agonist is combined with clusters selected from Chapter 2 to more effectively target the ligand to APCs. Combining the results of Chapters 2 and 3, **Chapter 4** describes the synthesis of peptide conjugates in which the TLR agonist, the CLR targeting mannoside clusters, and a peptide antigen are incorporated. These peptides are evaluated for their ability to mature APCs and cross-present the antigen. Analogs of these conjugates in which amino acids, functionalized with an acid-stable *C*-mannoside is incorporated are the subject of **Chapter 5**. Both the synthesis of a *C*-mannosyl lysine building block and its use in the inline SPPS synthesis of peptides are described. The antigen-presenting capacities of these conjugates are assessed and compared to the *O*-mannose analogs. As an alternative to peptidic mannoside carriers, **Chapter 6** describes the synthesis of glycosylated benzene tri-amides (BTAs) that can self-assemble into supramolecular fibers. Such systems could be considered in future work for a dynamic carrier for antigen and adjuvants. Finally, **Chapter 7** summarizes all the findings in this Thesis and discusses future directions that could be taken to follow up on data generated in this Thesis.

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