Synthetic carbohydrate ligands for immune receptors

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Chapter 1

General introduction

Introduction

Vaccination is one of the most successful approaches for the prevention of infectious diseases as it can provide a strong and lifelong immune response against pathogens. Classical vaccines are composed of attenuated or inactivated pathogens and owe their success mainly due to humoral immune responses. Vaccines for diseases such as cancer and HIV cannot be attained by analogous procedures and their development has proven to be more challenging as cellular immune responses are required.[1] Promising strategies to generate cancer vaccines exploit cancer specific peptides, the so-called neoepitopes[2] or tumor-associated carbohydrate antigens (TACAs)[3]. Peptide (neo)epitopes can be presented to T lymphocytes (T cells) via major histocompatibility complexes class I (MHC-I) or class II (MHC-II), present on antigen presenting cells (APCs). Both MHC classes present their antigen to a T cell: MHC-I activates a cytotoxic T cell (CTL) and MHC-II engages T helper (Th) cells. CTLs can eradicate for instance (virus-) infected cells or cancerous cells, while Th cells play an important role in generating a humoral (B cell) and cellular (T cell) responses, as they secrete cytokines resulting in the

activation and proliferation of B cells and CTLs (Figure 1). To achieve an effective CTL activation and thus anti-tumor immunity, T cells require three signals for activation.[4,5] Antigen presentation by the MHC on a target cell is the first signal, followed by interaction with co-stimulatory receptors on T cells and their corresponding ligands on APCs. The secretion of cytokines, such as interleukins (ILs), is considered to be the third signal. These three signals are important as immune tolerance can occur when antigen presentation is not followed by the last two signals.[6,7] Although TACAs are uniquely or overexpressed glycans on tumor cells, the deployment of these carbohydrates for cellular immune responses requires the assistance of a Th peptide epitope as carbohydrates are poorly immunogenic and only bind to B cells. For an effective anti-tumor immune response, B cells require the help of Th cells.

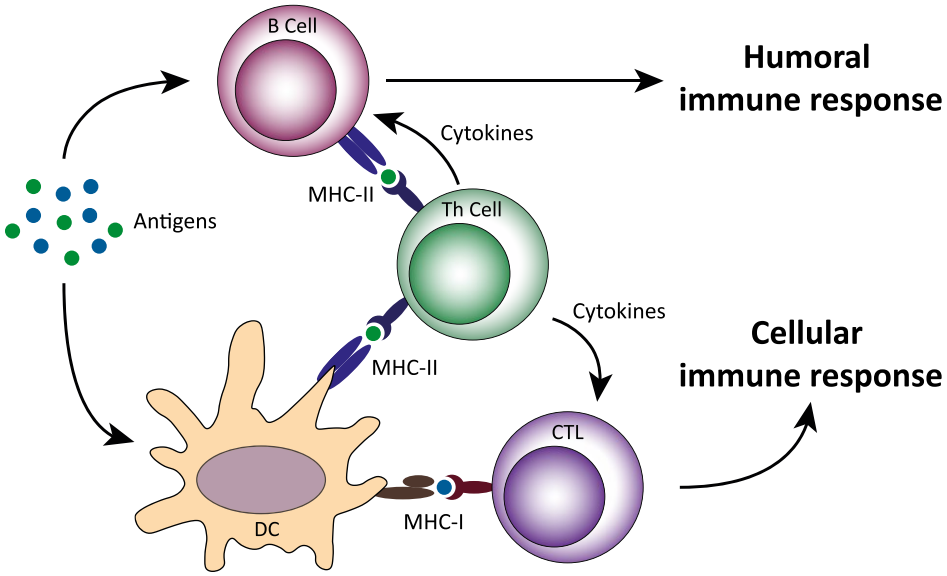


Figure 1. Schematic overview of acquiring a cellular and a humoral immune response.

Targeting antigen presenting cells

Antigen presenting cells, such as dendritic cells (DCs) play an important role in providing the previously mentioned three signals to obtain a T cell mediated immune response. To rapidly detect invading pathogens and send out “danger signals”, DCs are equipped with a row of pattern recognition receptors (PRRs).[8,9] PRRs can be divided into four main families of proteins: Toll-like receptors (TLRs), Nucleotide binding oligomerization domain (NOD)-like receptors (NLRs), C-type lectins and retinoic acid-induced gene (RIG)-I-like receptors. The binding of pathogen-associated molecular patterns (PAMPS) to a PRR results in the maturation of the DC and subsequent secretion of cytokines, the

so called “danger signal”. TLRs[10,11] are transmembrane glycoproteins (Figure 2), which are either located on the outer cell membrane (TLR1, 2, 4, 5, 6, 10, 11, 12) or expressed in endosomes (TLR3, 7, 8, 9, 13). The PRRs located on the outer membrane are able to recognize bacterial and fungal components such as lipomannan, lipoteichoic acids, di- and tri-acetylated bacterial lipopeptides, lipopolysaccharides (LPS) and flagellin, whereas the ones expressed in endosomes recognize viral or microbial nucleic acids, for example ssRNA, dsRNA, and the CpG motif. Most TLRs can be found as homodimers, with the exception of a few heterodimers: TLR1/TLR2, and TLR2/TLR6. In humans, TLRs 1-10 are expressed, while in mice TLR1-9 and TLR11-13 are found. NOD-like receptors (NOD1 and NOD2)[12] are intracellular proteins that can provide an innate immune response upon detection of components of the bacterial peptidoglycan. C-type lectins[13,14] and RIG-I-like[15] receptors recognize a diverse set of carbohydrate structures and viral RNA, respectively and will not be discussed further as they are beyond the scope of this Thesis. Another group of receptors present on APCs are the Fc receptors (FcRs), which form a bridge between the humoral and the cellular immune system.[16,17] FcRs are able to recognize immune complexes (ICs), which are formed from antibodies bound to antigens, and internalize the complex via the endocytic pathway resulting in both antigen presentation and DC-maturation and thus the secretion of cytokines.

Vaccination approaches

Vaccination with solely CTL or Th epitopes is not an effective approach to induce a cellular immune response.[18] Small peptides are generally poorly immunogenic and are unable to activate the innate immune system, which may lead to tolerance.[6,7] This problem can be obviated by the application of adjuvants (Figure 2).[19,20] Two types of adjuvants[1] exist, the first of which is involved in the improvement of the delivery of the antigen to DCs, for example liposomes, virosomes, emulsions and mineral salts.[21] The most commonly used adjuvant in vaccine formulations is Alum, that is able to enhance the potency of bacterial vaccines, but lacks the ability to induce a cellular immune response.[22] The second type of adjuvants are immunostimulants, comprising PRR-ligands that can induce a danger signal, such as the production of cytokines, by binding to, for example, one of the PRRs present on APCs.

This chapter describes selected well-defined synthetic ligands that are used as immunostimulants in vaccine formulations. Next, the so-called self-adjuvanting vaccines or conjugates with a specific antigen covalently bound to one or more immunostimulants, are discussed (Figure 2).[23,24] Self-adjuvanting vaccines are promising in inducing effective anti-tumor immunity. Finally, antibody-recruiting

molecule (ARM) strategies will be discussed as another approach to attain long-lasting adaptive immunity.[25]

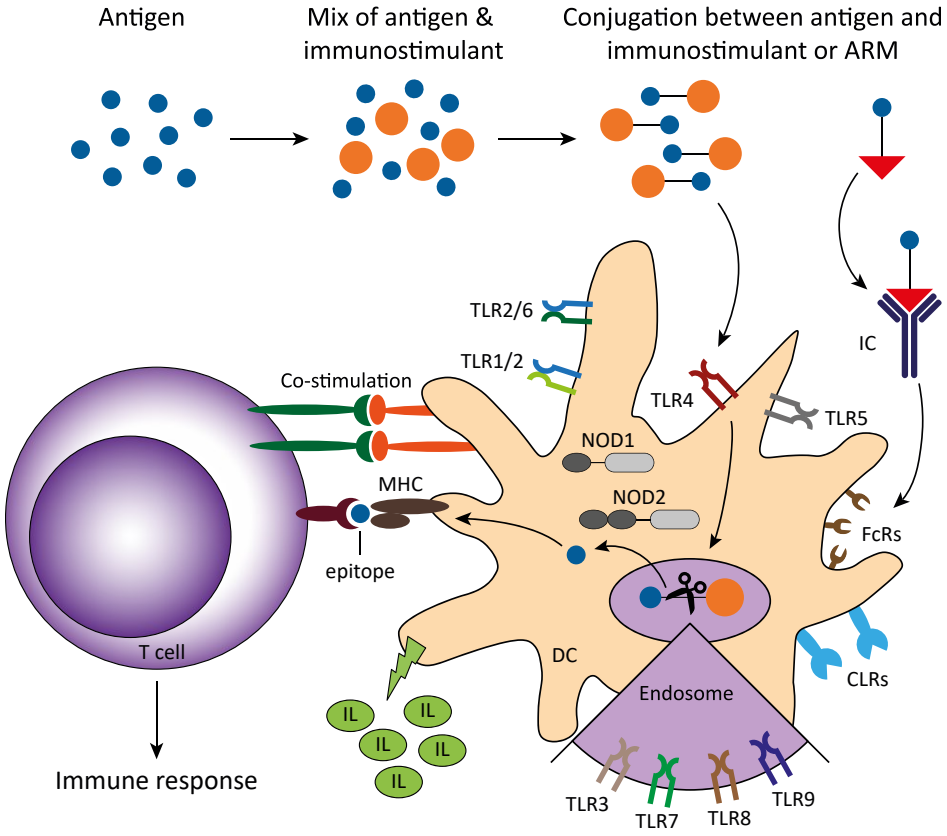


Figure 2. Schematic overview of acquiring an immune response using a conjugate by targeting DCs.

Immunostimulants

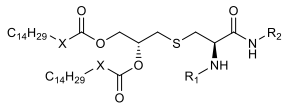
The discovery of well-defined (synthetic) PRR-ligands enables the study of structure-activity relations and the development of synthetic vaccine modalities.[11] Table 1 provides an overview of such TLR and NLR ligands that can be used as immunostimulating agents.

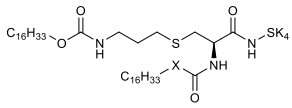
PRR	Natural ligands	Pathogen source	Synthetic ligands
TLR1/ TLR2	Triacylated lipopeptides	Bacteria	Pam_3C Pam_3CSK_4
TLR2/ TLR6	Diacylated lipopeptides	Bacteria	Pam_2CSK_4 MALP-2 FSL-1
TLR4	LPS	Gram negative bacteria	Lipid A MPL GLA AGPs
TLR7-8	ssRNA	Viruses	Imiquimod Resiquimod, 8-oxo-adenine derivatives
TLR9	CpG DNA	Bacteria, viruses	CpG ODN
NOD1	Meso-DAP	Bacteria	D-*i*E-DAP derivatives
NOD2	MDP	Bacteria	Muramyl dipeptide derivatives

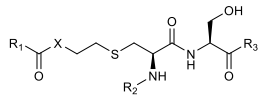
Table 1. Overview of synthetic well-defined TLR and NLR agonists.

TLR2 recognizes a wide variety of lipopeptides and lipoproteins and its specificity depends on the dimerization with either TLR1 or TLR6 as shown by the crystal structures of heterodimers TLR1/TLR2[26] and TLR2/TLR6.[27] Triacylated lipopeptides, such as Pam_3CSK_4 (**1**, Figure 3), one of the most potent TLR2 agonists to date, target TLR1/TLR2, as two lipid chains are inserted into the TLR2 pocket, while the amide-bound lipid is inserted into the TLR1 pocket. The amide-bound lipid chain also prevents the triacylated ligands from binding to TLR2/TLR6 as the hydrophobic pocket is blocked by bulky side chains. Pam_2CSK_4 (**2**) lacks the amide-bound lipid and triggers the dimerization between TLR2 and TLR6. Other diacylated lipopeptides, MALP-2 (**3**) and FSL-1 (**4**), are derived from *Mycoplasma fermentans* and *Mycoplasma salivarium* respectively and only differ in peptide composition.[28–31] The use of Pam_3CSK_4 TLR1/TLR2 agonists can improve the immune response by the production of cytokines, and its use has been shown to halt the development of cancer and can even induce tumor regression.[32] While it was shown that biological activity of Pam_3CSK_4 originates from the diasteroisomer having the RR-configuration, a diastereoisomeric mixture of Pam_3CSK_4 **1** is often employed because of synthetic ease and commercial availability. Several groups have tried to enhance the potency of TLR2 agonist **1**. A library of Pam_3CSK_4 derivatives in which the α-CH_2 of the amide lipid was replaced with an NH to form an extra hydrogen bridge with the receptor resulted in several new potent TLR2 agonists.[33] Others found that the immunological

properties strongly depend on the length of the lipid and the presence of *S-2*(*R*)-dihydroxypropyl-(*R*)-cysteine.[34–36] A chemically and metabolically more stable TLR2-ligand was made by replacing the two ester-linked palmitoyl groups with a 14-carbon chain via a carbamate linkage. The resulting ligand, SUP3 (**5**), was shown to induce a stronger antitumor response than **1**, when co-administered with different antigens.[37] One of the disadvantages of **1** is its poor solubility, and therefore Du *et al*. have generated more water soluble diacylated TLR2 agonists based on Pam_3CSK_4 introducing carbamate linkages as in **6**, which was shown to be as potent as Pam_3CSK_4.[38] The subtle change, replacing the α-CH_2 of the amide lipid in **6** for an NH (**7**), was shown to alter the binding preference of **6**/**7** from TLR1/TLR2 to TLR2/TLR6 binding. Monoacylated agonists, such as **8** and **9**, have been synthesized as well in an effort to improve the physical properties, e.g. water solubility.[39,40]







1 R_1 = $C(O)C_{15}H_{31}$, R_2 = SK_4, X = CH_2
2 R_1 = H, R_2 = SK_4, X = CH_2
3 R_1 = H, R_2 = GNNDESNISFKEK, X = CH_2
4 R_1 = H, R_2 = GDPKHPKSF, X = CH_2
5 R_1 = $C(O)C_{15}H_{31}$, R_2 = GK_3, X = NH

6 X = CH_2
7 X = NH

8 X = O, R_1 = $C_{15}H_{31}$, R_2 = H, R_3 = OMe
9 X = NH, R_1 = $OC_{16}H_{33}$, R_2 = Ac, R_3 = $NH(CH_2)_3N(CH_3)_2$

Figure 3. TLR2-ligands **1**-**9**.

TLR4 is the TLR that was first discovered in humans and it has been extensively studied. It has been shown that a complex of the lipopolysaccharide ligand and MD-2 binds to TLR4, upon which activation of the TRIF and MyD88 signaling pathways is induced. Notably, no other TLR is able to trigger both pathways.[41] TLR4 recognizes lipopolysaccharides (LPS), which are components of the Gram-negative bacteria cell membrane, and its endotoxic principle, lipid A (**10**, Figure 4), is the natural ligand for this receptor. Crystal structures have shown that six lipid chains are optimal for TLR4 activation as five are buried in the MD-2 pocket, while the remaining lipid is engaged in a hydrophobic interaction with TLR4.[42] Many groups have focused on the synthesis of lipid A agonists.[43–45] Removal of the phosphate on the anomeric position has led to the development of monophosphoryl lipid A (MPLA) derivatives, which are 1000-fold less toxic compared to lipid A and thus suitable as a vaccine adjuvant.[46,47] One of these MPLA analogues, MPL (**11**), has been used as a component of the AS04 adjuvant mixture in approved vaccines for hepatitis B and HPV.[48–50] A library of synthetic aminoalkyl glucosamine 4-phosphates (AGPs) has been developed, where the acetylated monosaccharides mimic the structure of lipid A.[51] These AGPs are not only easier to

synthesize, they also induce comparable or even enhanced immunostimulatory activities.[52] One of these AGPs, RC-529 (**12**) has been shown in human clinical trials to have an excellent safety profile. In an effort to mimic the diphosphate nature of lipid A, Lewicky *et al*. synthesized various analogues of **12** and biological evaluation of these compounds showed that **15**, which features an additional carboxylic acid moiety, has a higher potency than **13** and **14**.[53] Another structure-activity relationship study of AGPs showed that the potential of this class of agonists also relies on the length of the secondary acyl chains and the nature of the functional group on the aglycon component.[54,55] CRX-527 (**17**), wherein the L-serinyl carboxylic group mimics the anomeric phosphate of lipid A, was shown to be more potent than CRX-524 (**16**). Whereas CRX-527 (**17**) induces the production of MyD88- and TRIF-dependent cytokines, CRX-547 (**18**), containing a D-serinyl carboxylic group, was shown to be TRIF-selective.[56] TLR4 agonists, such as **19**[57] and **20**[58], with no structural similarity to lipid A also exist, and the latter has been used in vaccine modalities in combination with other TLR ligands.[59,60]

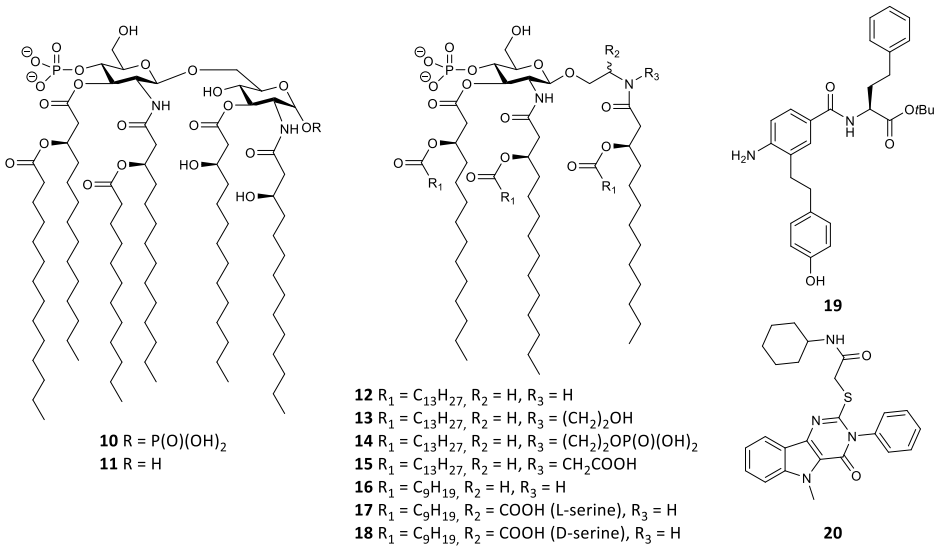
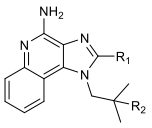


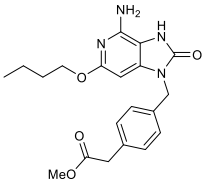
Figure 4. TLR4-ligands **10**-**20**.

TLR7 and TLR8 bind to single stranded viral RNA[61] and they have been the subject of studies to arrive at small molecule agonists (Figure 5).[62] The synthetic ligands for these TLRs are based on imidazoquinolines, for example imiquimod (**21**), resiquimod (**22**), and 8-oxo-adenine derivatives, such as **23** and **24**, and these ligands have been shown to have promising adjuvant properties.[63–65] A cream with imiquimod, suitable for external

use, was shown to enhance the anti-ovalbumin antibody response in mice 100-fold compared to mice that were not given the adjuvant.[66] Bacterial DNA and synthetic oligodeoxynucleotides with an unmethylated CpG motif (CpG ODNs) are able to trigger an immune response via TLR9.[67] Vaccines containing the synthetic CpG adjuvant have been tested in preclinical studies and have shown to enhance both humoral and cellular immune responses.[68] Moreover, simultaneous administration of this adjuvant and antigens proved to be crucial to obtain significantly enhanced antibody response in a hepatitis B vaccine.[69] Phase II trials with A15 (a mixture of MPL, QS-21 and CpG 7909) have demonstrated to be a promising in the treatment of MAGE-A3 melanoma.[70,71]



21 R_1 = H, R_2 = H
22 R_1 = $CH_2O(CH_2)_2$, R_2 = OH



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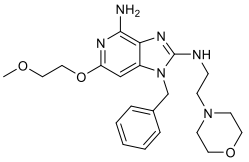

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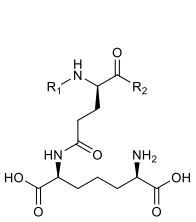
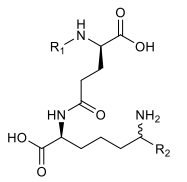
Figure 5. TLR7/8-ligands **21**-**24**.

NOD1 and NOD2, the founding members of the NOD-like receptors (NLRs) family, are able to recognize components of the bacterial peptidoglycan (PG). D-Glutamyl-meso-diaminopimelic acid, *i*E-DAP (**25**, Figure 6), has been found to be the minimal structure required for interaction with NOD1.[72] Several structure-activity relationship studies have been performed to determine what modifications on **25** are tolerated and what has to be done to increase the potency of **25**, as NOD1 ligands are generally relatively poor immune stimulatory agents. It was found that elongation with L-Ala (**26**) increased the activity of the ligand, whereas replacing glutamic acid with glutamine (**27** and **28**) decreased the NOD1 activity.[73,74] Masumoto *et al*. showed that increasing the lipophilicity of the ligand improved the immune response as the induced NOD1-dependent NF-κB activation was several 100-folds higher for **29** and **30** compared to **25**.[73] Substitution of the meso-diaminopimelic acid component of **31** with for example L-serine (**32**) was found to reduce the NOD1-agonistic activity.[75] The minimal structure that is capable of triggering NOD2 activation is muramyl dipeptide (MDP, **33**). Several groups have investigated the influence of modification at the 2-amine of MDP and a glycolylated MDP, for example **34**, was demonstrated to be more potent than **33**, containing an *N*-acetyl group.[76–78] MDP **34** has also been shown to be more efficacious in the induction of an ovalbumin specific T cell response.[76] Increasing the lipophilicity of MDP by monoacylation at the 6-*O* position with decanoic acid (**35**) or stearic acid (**36**)

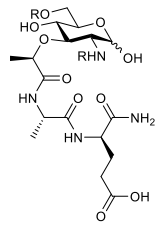
increased the activity of the ligand, while 4,6-diacetylation did not. Notably, the activity of these MDP derivatives was originally shown to originate from TLR2 and TLR4 activation rather than interaction with NOD2. Willems *et al*. have recently shown that lipophilic MDP derivatives can also act in a TLR2-independent manner.[79,80] Conjugation of MDP via its 6-*O* position with TLR2-ligand Pam_2Cys, led to dual adjuvant **37**, which enhanced the immune response compared to a mixture of the separate ligands and co-administration of **37** and a model antigen led to the induction of high antigen-specific IgA and IgG titers.[81] Mifamurtide (**38**), another conjugate between MDP and Pam_3Cys, was found to be effective against osteosarcoma and has been approved as a drug against bone cancer.[82]



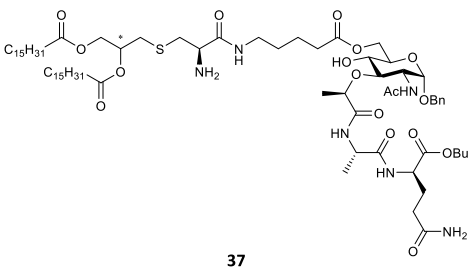
25 R_1 = H, R_2 = OH
26 R_1 = H, R_2 = OH
27 R_1 = L-Ala, R_2 = NH_2
28 R_1 = L-Ala, R_2 = NH_2



29 R_1 = $C(O)C_{14}H_{29}$, R_2 = COOH
30 R_1 = $C(O)C_{15}H_{31}$, R_2 = COOH
31 R_1 = $C(O)C_{11}H_{23}$, R_2 = COOH
32 R_1 = $C(O)C_{11}H_{23}$, R_2 = H



33 R_1 = $C(O)CH_3$, R_2 = H
34 R_1 = $C(O)CH_2OH$, R_2 = H
35 R_1 = $C(O)CH_3$, R_2 = $C(O)C_9H_{19}$
36 R_1 = $C(O)CH_3$, R_2 = $C(O)C_{17}H_{35}$



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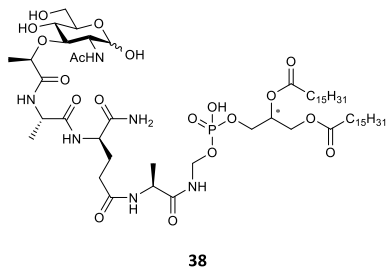

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Figure 6. NOD1- and NOD2-ligands **25**-**38**.

Synergy

During an infection, a broad range of PRR-ligands are presented to the immune system which are recognized by the diverse PRRs. Immunization with different synthetic PRR-ligands can act synergistically resulting in an enhanced immunostimulatory effect and the synergistic activity of different PRR-ligands has therefore been the subject of many studies.[60,83–87] Tada *et al*. combined synthetic ligands for NOD1 and NOD2 with TLR2, TLR3, TLR4, and TLR9 ligands and found that the combination of lipid A with either MDP or FK565, an *i*E-DAP analogue, enhanced the immune response more than 1000-fold than each separate stimulant.[85] Co-stimulation with **1** (TLR2-ligand) and **31** (NOD1-

ligand) was shown to enhance the proliferation, expansion, and effector function of T cells.[88] Conjugation of two or more PRR-ligands has also been investigated, besides the previously mentioned combinations of NOD2 and TLR2 (**37** and **38**), MDP has been conjugated to lipid A analogues.[81,89,90] Synergistic effects can also become problematic and the combination of LPS and MDP has led to lethal outcomes in mice.[91]

Conjugation of antigens and immunostimulants

Our understanding of the innate and the adaptive immune system continuously grows, enabling the development of anti-cancer vaccines with enhanced immunological properties. While the administration of a mix of anti-tumor antigens and immunostimulants have led to promising results, the immunogenicity of a vaccine can even be further enhanced by the conjugation of the antigens to an immunostimulant. These "self-adjuvanting" vaccines ensure the simultaneous delivery of both components to APCs, such as DCs, thereby inducing a stronger humoral and cellular immune response.[23,92,93] As a result, the required dose can be lowered which reduces the chance of possible (toxic) side effects. The following sections of this Chapter describe a number of selected conjugates targeting APCs with the goal to either up-regulate the production of cytokines and co-stimulatory molecules or to increase the uptake of the antigens.

TLR2 based conjugates

Due to its synthetic ease and commercial availability, TLR2-ligands have been used in peptide-conjugates since 1989. Immunological evaluation of conjugate **39** (Figure 7) demonstrated for the first time the possibility of inducing an influenza virus-specific CTL response in mice.[94] This set the stage for the conjugation of other Pam_3C analogues such as Pam_3CSK_4 (**1**), which was conjugated to antigenic peptides derived from ovalbumin containing the MHC-I (SIINFEKL) and MHC-II (ISQAVHAAHAEINEAGR) epitopes in several studies.[95–97] First, Khan *et al*. synthesized conjugate **40** via solid phase peptide synthesis (SPPS) using FMOC/HCTU chemistry.[95] The conjugate induced an enhanced T cell specific response due to improved antigen presentation and DC maturation. It was also discovered that the uptake of the antigens occurred independently of TLR expression. Next, Khan *et al*. investigated the immunological behavior of the two Pam_3C diastereoisomers.[96] The IL-12 production *in vitro* was significantly higher for the *R*-conjugate **41** as compared to that of the *S*-isomer **42** indicating that the *R*-isomer induces better activation of DCs, which confirms previous studies on the chirality of Pam_3C.[98,99] However, the racemic mixture **43** was shown to be as potent as **41** in the production of IL-12 and it demonstrated an enhanced CTL

response *in vivo*. Next, the TLR2-ligand peptide conjugates **44** and **45**, in which **1** was conjugated to Th epitopes derived from ovalbumin (**44**) and the Moloney virus envelope (**45**) were studied.[97] *In vivo* experiments with **43**-**45** show enhanced CTL and Th cell responses and more efficient anti-tumor immunity when the TLR2-ligand is covalently bound to the antigen in comparison to a mixture of the ligand and the corresponding antigen. These results demonstrate the potential of PRR-ligand-peptide conjugates as both CTL and Th cell priming are necessary for cancer immunotherapy. These finding were therefore exploited in the GMP synthesis of **46** and **47**, which are used for vaccination against human papillomavirus (HPV) type 16. Herein, UPam[33], an improved Pam_3C analogue, was conjugated at the *N*-terminus of the antigenic peptide via SPPS.[100] The conjugated synthetic long peptides (SLPs) were shown to be efficiently processed by APCs and significantly enhanced the *ex vivo* stimulation of lymph node-derived T cells. The Pam_3C conjugates (**43**, **44**, **48**) were compared with the corresponding UPam analogues (**49**-**51**) to show that the latter are more potent *in vitro* and *in vivo*. In combination with photodynamic therapy tumor eradication can be induced with these conjugates.[101] Besides antigenic peptides, TLR2-ligands have also been conjugated to carbohydrate antigens, TACAs.[102] Several groups have reported the synthesis of a multicomponent vaccine, in which a TLR2-ligand is covalently bound to a B cell epitope and/or a T cell epitope.[103–108] In 2005, the group of Boons used this strategy for the development of a three component vaccine, wherein Pam_3C is conjugated to a Th epitope (YAF) and a tumor-associated Tn-antigen.[109] The latter is a B cell epitope that is overexpressed on the surface of human cancer cells. The conjugate suffered from poor solubility and conjugate **52** elicited only low titers of IgG antibodies. An improvement of this conjugate was made by replacing Pam_3C by the more potent Pam_3CSK_4 (**53**).[110] Conjugation of this ligand to a helper T cell epitope (KLFAVWKITYKDT) derived from poliovirus and a MUC1 glycopeptide B cell epitope elicited excellent high titers of IgG antibodies in mice, while its Pam_2CSK_4 analogue (**54**) gave a low immune response. The group of Kunz investigated the impact of multivalent glycopeptide antigens on the immunogenicity of a vaccine. To this end, Pam_3CSK_4 was either conjugated via SPPS or click chemistry to one, two, or four MUC1 glycopeptides containing either two Tn antigens or one STn antigen (**55**-**60**).[111] Immunological evaluation showed **60** to be a promising vaccine modality as it induced efficient killing of tumor cells.[112]

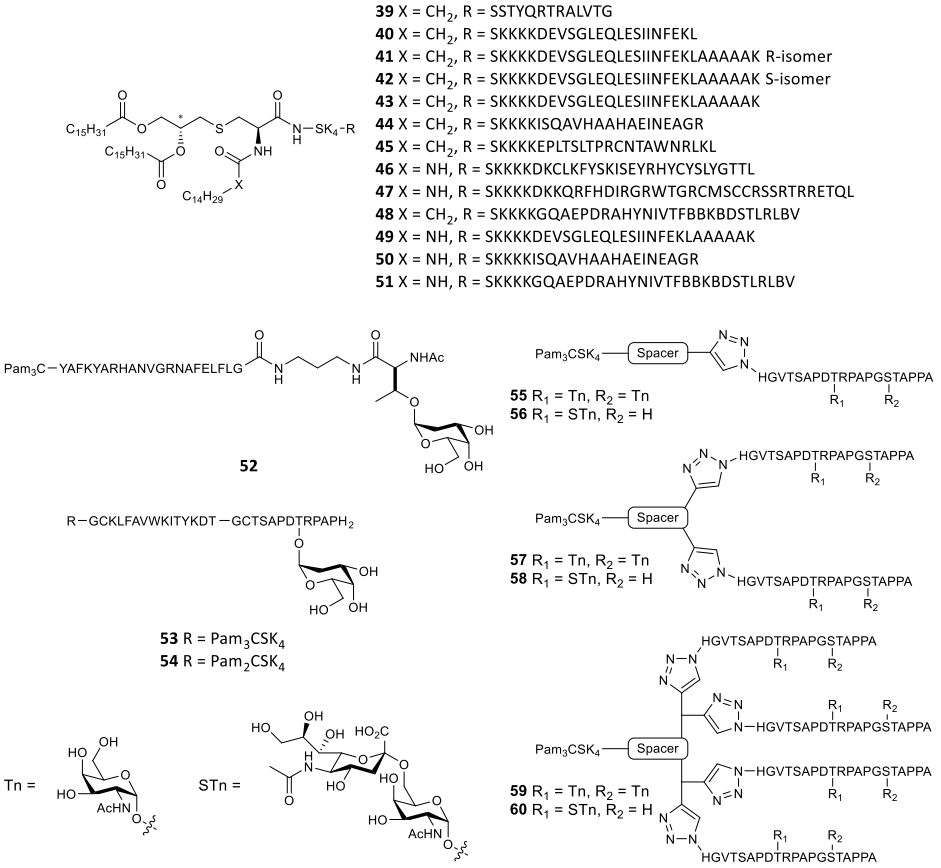


Figure 7. TLR2-conjugates **39**-**60**.

TLR4 based conjugates

Although many groups have studied the adjuvanting effect of TLR4-ligands, conjugates with a TLR4-ligand are scarce, probably because these glycolipids are not only difficult to synthesize but also the coupling to an antigen is challenging. Up to now, there is only one example of a conjugate between a protein and TLR4-ligand.[113] Schülke *et al*. coupled the detoxified TLR4-ligand, MPLA (**11**), to ovalbumin via a carbamate linkage and the resulting conjugate induced a stronger immune response compared to a mixture of unconjugated **11** and ovalbumin. Although TLR4-ligands peptide-conjugate have not been reported (See Chapter 2 of this Thesis), they have been conjugated to TACAs.[114,115] Ziaco *et al*. developed a semisynthetic strategy to obtain MPLA derivatives equipped with clickable conjugation handles (Figure 8).[116] Herein, **11** was obtained via a semisynthetic method and then subjected to a regioselective oxidation of the primary

alcohol. The carboxylic acid could then be used to install the linker. To demonstrate the potential of the clickable MPLA, they synthesized two conjugates with a Tn (**61**) and a TF antigen (**62**). Preliminary evaluation showed that the conjugates could successfully induce cytokine production. The group of Guo developed a synthetic approach to conjugate MPLA to TACAs via the anomeric position of the reducing end sugar.[117] To this end, MPLA from *Neisseria meningitidis* was elongated with a small linker and coupled to GM3 or a GM3 derivative yielding conjugate **63** and **64** respectively. Due to solubility issues, pure conjugates **63** and **64** could not be used for immunization of the mice and therefore liposomes containing the conjugates were prepared. Immunological studies showed that **64** elicited a strong immune response with the total antibody titer four times higher than that of **63**. In a follow-up study, they synthesized conjugates **65**-**68** in which MPLA is coupled via the same strategy as before to sTnNPhAc, a modified TACA.[118] All four conjugates were incorporated in liposomes and these provided a similar immune response pattern. In the series **66** was shown to be the best vaccine as it elicited the highest response and was more consistent in the production of the total amount of antibodies. Conjugate **65** was significantly less potent than **66** and **67**, demonstrating that the hydroxyl functions on the lipids play an important role in receptor binding. On the other hand, the length of the lipids and the incorporation of an additional lipid chain had a relatively small impact. Conjugation of MPLA to a Globo H antigen (**69**) provided a vaccine modality that not only induced more IgG antibodies than the corresponding KLH conjugate, it also resulted in a faster immune response.[119]

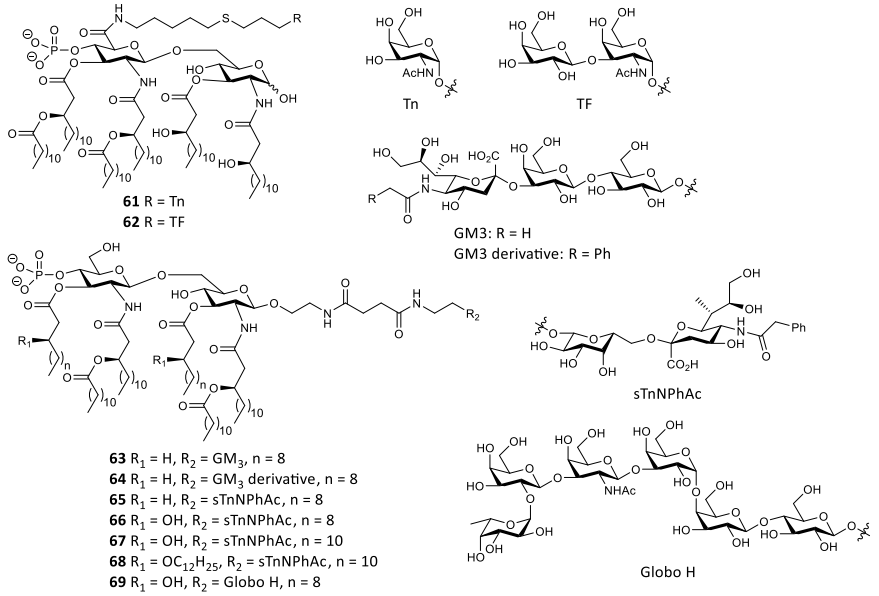
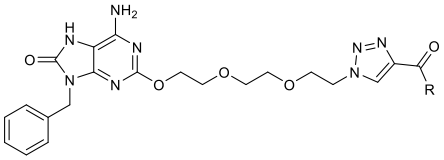


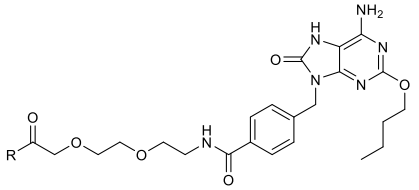
Figure 8. TLR4-conjugates **61**-**69**.

TLR7/8 based conjugates

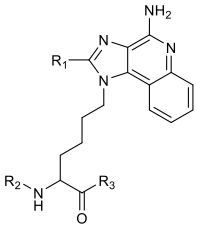
Agonists for TLR7 and TLR8 have received considerable attention in conjugation chemistry because their chemical structure presents multiple sites for functionalization. Besides their use in a mixture with a protein or conjugated to proteins[120–122], they have also been covalently linked to antigenic peptides. Weterings *et al*. combined SPPS and Cu(I) catalyzed Huisgen cycloaddition for the conjugation of 2-alkoxy-8-hydroxy adenine (TLR7-ligand) to ovalbumin-derived peptides DEVSGLEQLESIINFEKL and DEVSGLEQLESIINFEKLAAAAAK, that both contain the MHC-I epitope SIINFEKL.[123] Although improved antigen presentation was detected after stimulation of DCs with conjugates **70**-**73** (Figure 9), the conjugates lacked the ability to activate DCs as almost no IL-12 was produced. These results show how important the right conjugation site of an agonist can be, as conjugation via the benzyl moiety did result in DC maturation.[121] These findings led to the design of a TLR7-ligand, extended on the benzyl moiety, and its application in conjugates **74**-**76**, that were able to induce DC maturation.[124] T cell proliferation experiments not only showed that the conjugates perform better than a mixture of peptide and TLR7-ligand, but also that *N*-terminus conjugates (**74** and **75**) perform better than the *C*-terminus conjugate (**76**). The group of Taguchi synthesized a series of synthetic TLR7-ligand amino acids containing the imidazoquinolyl structure (**77**-**81**) of which **81** was shown to be the most potent agonist.[125] Ligand **81** was therefore selected for conjugation to either the *N*-terminus (**82**), the *C*-terminus (**83**) or both (**84**) of a peptide derived from the influenza A virus M2 protein. Immunological evaluation showed that the obtained conjugates of **81** exhibit poor adjuvanting properties.



70 R = DEVSGLEQLESIINFEKL
71 R = DEVSGLEQLESIINFEKLAAAAAK (N-terminus)
73 R = DEVSGLEQLESIINFEKLAAAAAK (C-terminus



74 R = DEVSGLEQLESIINFEKL
75 R = DEVSGLEQLESIINFEKLAAAAAK (N-terminus)
76 R = DEVSGLEQLESIINFEKLAAAAAK (C-terminus



77 R_1 = H, R_2 = H, R_3 = OH
78 R_1 = CH_3, R_2 = H, R_3 = OH
79 R_1 = C_2H_5, R_2 = H, R_3 = OH
80 R_1 = C_3H_7, R_2 = H, R_3 = OH
81 R_1 = C_4H_9, R_2 = H, R_3 = OH
82 R_1 = C_4H_9, R_2 = H, R_3 = SLP
83 R_1 = C_4H_9, R_2 = SLP, R_3 = H
84 R_1 = C_4H_9, R_2 = SLP, R_3 = SLP

SLP = SLLTEVETPIRNEWGCRCNDSSD

Figure 9. TLR7/8-conjugates **70**-**84**.

TLR9 based conjugates

Conjugation of CpG oligonucleotides (ODN) to protein antigens has been well-studied and these conjugates have been shown to enhance the immunogenicity of the antigens.[68,120,126–128] The group of Diamond synthesized a library of conjugates, combining CpG with several minimal CTL and Th epitopes.[129] These TLR9-mediated self-adjuvanting vaccines were superior in cytokine production and protection against viral infection compared to non-covalently linked mixtures of the corresponding molecules. Khan *et al*. conjugated CpG to antigenic peptides comprising the MHC-I epitope SIINFEKL and compared the resulting conjugates **85** and **86** to conjugates containing the non-stimulatory oligonucleotide GpC, **87** and **88** (Figure 10).[95] While the SIINFEKL-specific T cell response of the GpC-conjugates **87** and **88** was equal to that induced by a mixture of peptide and adjuvant, the response obtained with **85** and **86** was significantly higher, showing that the T cell response depended on the activation of DCs. A three-component vaccine containing the TLR9-ligand CpG was made by the group of Boons. Herein, CpG was conjugated to a Th epitope and a MUC1 peptide, serving as a B cell epitope. Immunization with conjugates **89** and **90** did not result in significant improvement in anticancer properties, while its Pam_3CSK_4-analogue did, which demonstrates that the choice of build-in adjuvant can be important for the efficiency of a vaccine.

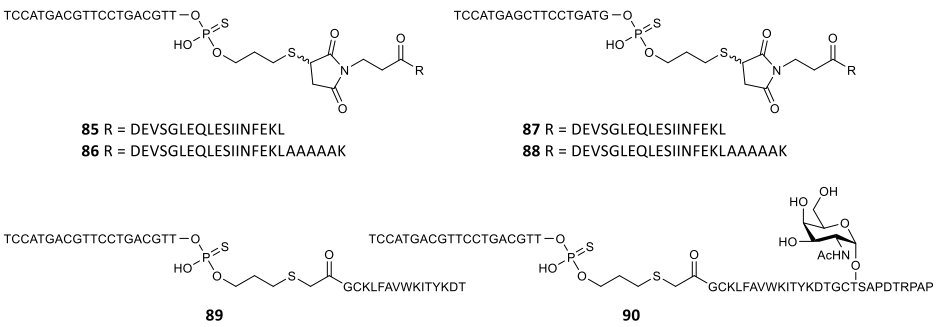


Figure 10. TLR9-conjugates **85**-**90**.

NOD2 based conjugates

In comparison to the TLR-family, NLR-ligands have been rarely used in a synthetic conjugation approach. Carelli *et al*. have coupled MDP to peptide fragments derived from the luteinizing hormone-releasing hormone (LH-RH). Besides the fact that these conjugates can potentially be used in the veterinary field for castration, they can also be exploited for the treatment of LH-RH dependent tumors.[130,131] In a three-component vaccine MDP was covalently bound to a B cell epitope derived from a growth hormone

and a T cell epitope derived from ovalbumin.[132] This conjugate was shown to produce high titers of antibody and an increased body weight for the immunized rats. Willems *et al*. reported the synthesis of a MDP building block, which is suitable for SPPS, and they generated conjugates, in which MDP was covalently connected to an ovalbumin derived model peptide containing the MHC-I epitope SIINFEKL.[133] Several conjugation sites were investigated by coupling MDP via the dipeptide to the *N*- or the *C*-terminus of the peptide giving **91** and **92** (Figure 11). Alternatively, the linker on the anomeric position of *N*-acetylglucosamine (E-MDP) was connected to the *N*- or the *C*-terminus of the same peptide giving **93** and **94**. According to the level of IL-12 production, MDP is a poor immunostimulator, while the antigen presentation induced by the conjugates was comparable to that induced by the Pam_3CSK_4 conjugate **43**. In a follow-up study[134], the synergistic acting of NOD2 and TLR2 was exploited by the assembly of bis-conjugates (**95**-**98**) containing MDP and the TLR2-ligand, Pam_3CSK_4.[85] Although all conjugates showed a strong IL-12 production, conjugate **96** proved to be the most potent in activating DCs. The latter conjugate also induced an enhanced CTL priming compared to the mono-conjugates containing either MDP or Pam_3CSK_4 indicating that these bis-conjugates could be of use for the treatment of virus infections or cancer.

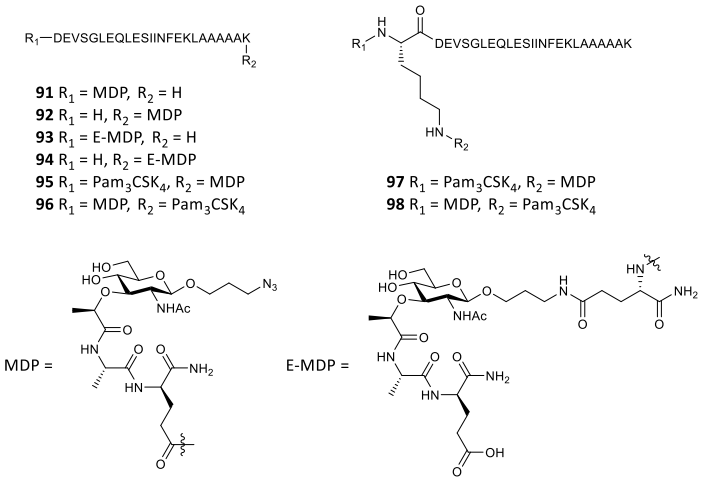


Figure 11. NOD2-conjugates **91**-**98**.

Antibody-recruiting molecules-based conjugates

Fc receptors (FcRs) on APCs are able to enhance the uptake of immune complexes, formed by antibody-recruiting molecules (ARM) bound to their designated antibody. The FcR-mediated uptake can also induce the production of cytokines resulting in enhanced processing and presentation of the antigens leading to an effective immune

response[135] and therefore ARM-conjugates have been investigated as a strategy to improve vaccines.[25] Several ARMs have been studied in vaccine formulations. Besides targeting APCs, ARM-conjugates can also be used to target tumor cells and recruit antibodies resulting in a localized cytotoxic immune response.[136–139] The group of Spiegel synthesized several bifunctional linkers (**99**-**101**, Figure 12), wherein 2,4-dinitroaniline was conjugated on one side and a target-binding molecule on the other side and could be used to either induce phagocytosis of fungi, the inactivation of HIV virus or the destruction of cancer cells.[140–142] Another ARM-strategy is based on the fact that virtually almost all people have endogenous antibodies against tetanus toxoid.[143,144] Thus, the B cell epitope of tetanus toxin, FIGITELKKLESKINKVF as part of a longer peptide, was conjugated via thiol-maleimide chemistry to SLPs containing CTL epitopes derived from either ovalbumin (**102**), cytomegalovirus (**103**) or influenza virus (**104**). The conjugates were able to induce DC and T cell activation as a result of improved antigen uptake. Anti-α-Gal antibodies represent 1-3% of all immunoglobulins and are produced by about 1% of all B cells, and these have also been explored in vaccines against HIV, lymphoma cells and influenza virus. The conjugation of α-Gal epitope (**105**) to either an HIV gp-peptide, tumor-specific antibodies or PR8 derived peptides was shown to enhance the immunogenicity of the vaccines.[145–149] One of the disadvantages of the α-Gal epitope in model vaccination studies is the need to use expensive KO mice. Chen *et al*. have demonstrated that L-rhamnose monosaccharides can be a good alternative since anti-L-rhamnose antibodies are not only one of the most abundant antibodies in humans[150,151], and wild-type mice can be used instead of KO mice.[152] Several studies exploited rhamnose-functionalized proteins[153] and liposomes[154–156] for cancer immunotherapy. Sarkar *et al*. synthesized a three-component vaccine consisting of rhamnose as ARM molecule, a Th cell epitope (YAF) and a tumor-specific antigen (Tn) using SPPS.[157] A T cell proliferation study showed that conjugate **106** was as active as conjugate **107** when using a 10-fold lower concentration, demonstrating that the addition of rhamnose-monosaccharide results in a better internalization, processing and presentation of the epitope.[157]

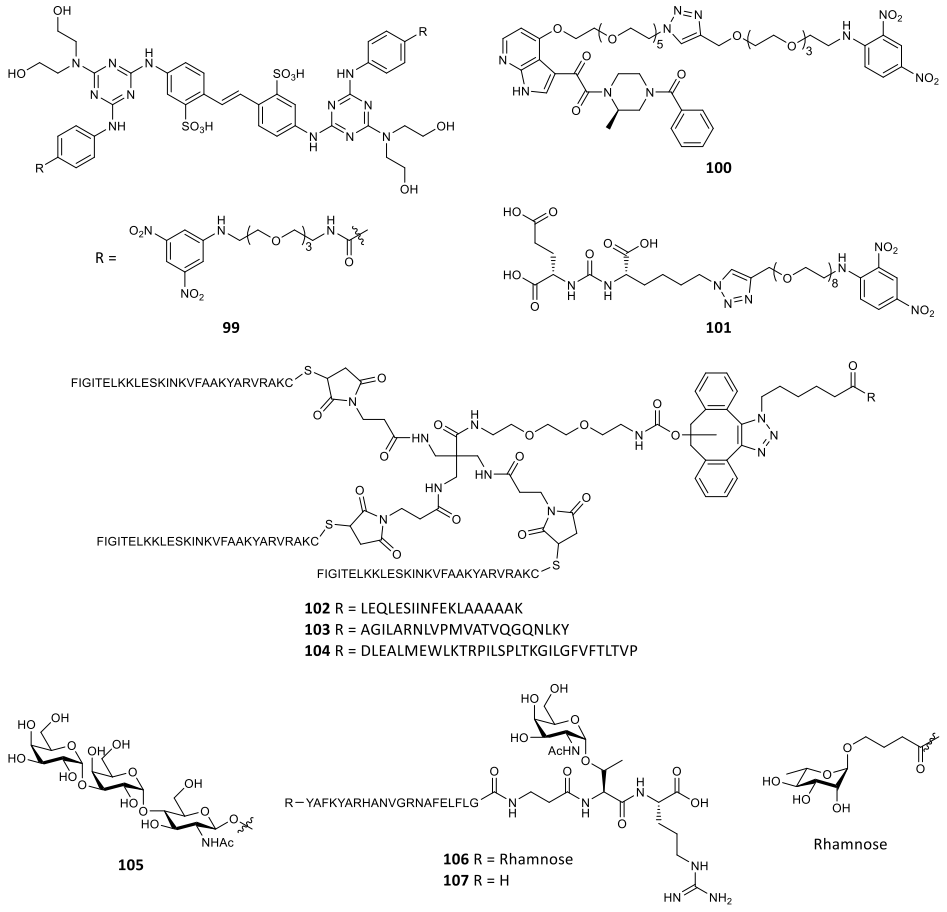


Figure 12. ARM-based conjugates **99**-**107**.

Outline of this thesis

The research described in this Thesis aims at the development of cancer vaccines by the design and synthesis of conjugates in which structurally defined adjuvants are covalently attached to antigenic peptides. **Chapter 1** has provided a concise overview of selected well-defined synthetic ligands that have been used as immunostimulants in vaccine formulations. **Chapter 2** describes the design, synthesis and immunological evaluation of four TLR4-ligands and four TLR4-ligand antigen conjugates. A new synthesis route towards (R)-3-alkyloxytetradecanoic acid is presented together with the optimized synthesis of the monophosphoryl lipid A analogue, CRX-527, in which the key step is the introduction of the lipid tails. Two different linkers between the CRX-527 ligand and an antigenic peptide are investigated and the CRX-527 ligand has been conjugated to either the *N*- or the *C*-terminus of an antigenic peptide via thiol-

maleimide-chemistry. The *in vitro* studies of the resulting ligands and "self-adjuvanting" conjugates showed that the choice of spacer type is critical to obtaining a proper immune response. The preparation of four bis-conjugates containing a NOD2-ligand and a TLR2-ligand as well as four mono-conjugates with only a NOD2-ligand is the subject of **Chapter 3**. Herein, two types of NOD2-ligands featuring either an *O*- or a *C*-MDP-moeity, with either an *N*-acetyl or an *N*-glycolyl substituent have been prepared. The *O*-MDP contains an azidopropanol spacer at the anomeric position of the glucosamine and it was covalently bound via its isoglutamic acid moiety to an antigenic peptide derived from the human papilomavirus using SPPS chemistry. The *C*-MDP derivatives were conjugated via the anomeric center of the glucosamine to the peptide using an online SPPS approach. **Chapter 4** covers the use of the mannose-6-phosphate receptor that could mediate a more efficient delivery of conjugates to the endosome to improve the immune response. To this end, two types of mannose-6-phosphonates building blocks, an *O*-analogue and a *C*-analogue, have been synthesized and conjugated to either a CTL or to a Th epitope using Cu(I) catalyzed 1,3-dipolar cycloaddition or SPPS chemistry. **Chapter 5** describes the synthesis of two *C*-rhamnose-lysine building blocks, which are suitable for SPPS chemistry. One, two, three or six *C*-rhamnose-functionalized lysines were linked at the *N*-terminus end of an antigenic peptide to investigate the multivalent effect on the binding to anti-rhamnose antibodies to obtain an improved vaccine based on the ARM-strategy. **Chapter 6** describes the synthesis of four different *C*-glycosyl functionalized lysines, the glycosidic linkage of which are stable against the acidic conditions used in SPPS. The building blocks were equipped with protecting groups that could be removed under acidic conditions, concomitantly with the cleavage of the synthesized peptides form the resin. In **Chapter 7**, the research of this Thesis is summarized and some future prospects are presented.

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