Design and synthesis of NLR and TLR based ligand-antigen conjugates

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Chapter 1

General introduction

1.1 Introduction

The defense mechanism of mammals can be divided into the innate and adaptive immune system. The adaptive immune system, also known as the specific immune system, is responsible for the prevention or elimination of pathogen growth amongst others. In order to do so, the adaptive immune system is activated by the innate immune system, also known as non-specific immune system. The innate immune system is the first line of defense that detect pathogens by specialized receptors, the pattern-recognition receptors (PRRs). These receptors are especially located in and on antigen presenting cells (APC) of the immune system such as dendritic cells (DCs). PRRs detect pathogen associated molecular patterns (PAMPs) that solely occur in microbes and not in mammalian cells. Relevant examples of PAMPs are bacterial lipopolysaccharide (LPS, **1**), nucleic acids (bacterial or viral DNA and RNA, CpG **2**), flagellar proteins, peptidoglycans (PG, **3**) and lipoteichoic acids (**4**) (*Figure 1*).

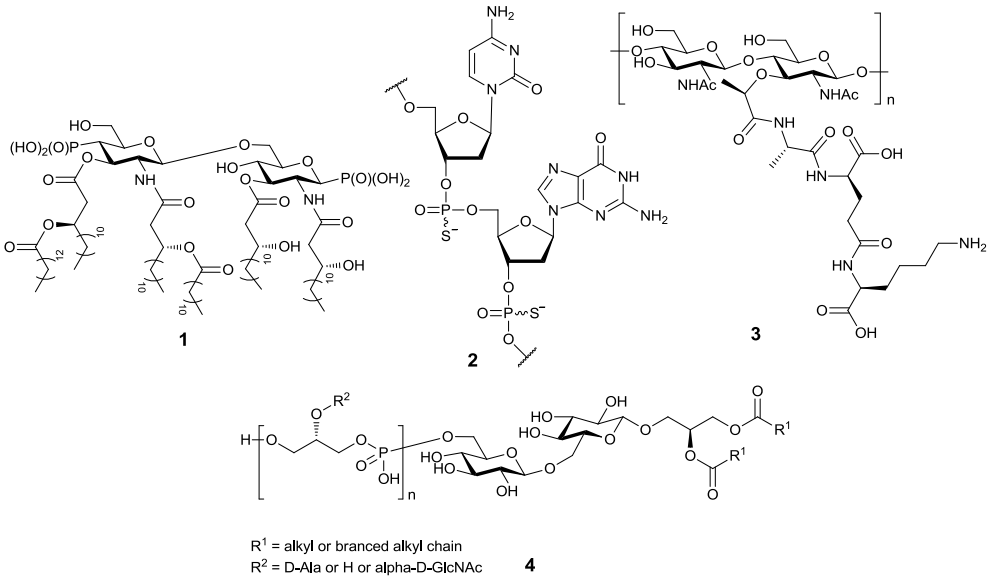
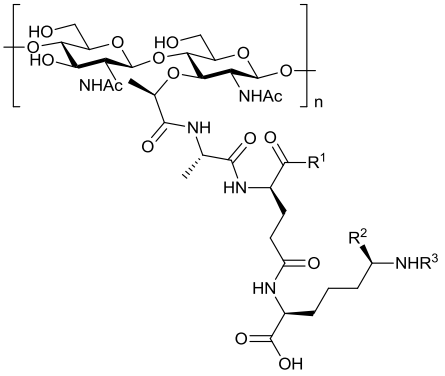


Figure 1. Some pathogen associated molecular patterns: LPS lipid A (**1**), nucleic acids (CpG DNA, **2**), peptidoglycan (**3**) and lipoteichoic acid (**4**).

The broad structural variety of PAMPs is reflected in the number and types of PRRs. The PRR family consists of Toll-like receptors (TLRs), nucleotide oligomeric domain like receptors (NLRs), retinoic acid-induced gene-I-like or RIG-like receptor (RLRs) and C-type lectin receptors (CLRs).[1] The Toll-like receptors (TLRs), the most studied PRRs, of which TLR 1 – 10 are found in human and TLR 1 – 9, 11 – 12 in mice, can be expressed on the cell surface or in endosomes of certain immune cells. In this Chapter special attention is paid to TLR2, located at the cell surface of immune cells. TLR2 receptors form heterodimers with either TLR1 or TLR6 and each combination recognizes distinct ligands. NLRs are not membrane bound and situated in the cytosol. In this Chapter special attention is paid to the NOD1 and NOD2 receptors.[2]

Freund was the first to recognize that samples originating from pathogenic bacteria can stimulate the immune system. He found that heat killed mycobacterium cells suspended in a mineral oil induced systemic immune activation in mammals. This discovery led to Freund's adjuvant, a frequently used immune stimulator. This activation is not restricted to mycobacterium, and samples derived from other bacteria exhibit similar abilities. With the discovery of PRRs it became clear that molecules originating from the cell wall of both gram-positive (**5**) and gram-negative (**6**) bacteria are recognized by specific PRRs. The TLR2 receptor detects lipopeptides and lipoproteins present in both bacterial types. The NOD1 and NOD2 receptors recognize fragments of the

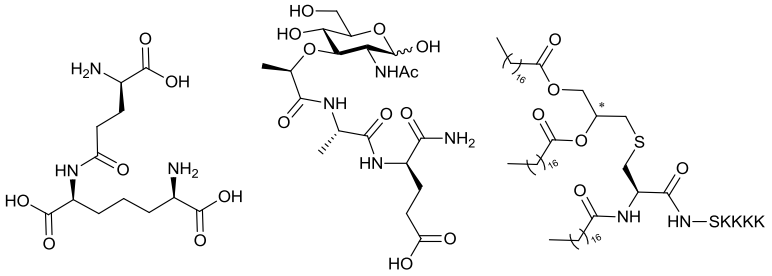
peptidoglycan (PG), an essential part of both types of the bacterial cell walls (*Figure 2*).[3-5]



5 : Gram positive PG: $R^1 = NH_2$, $R^2 = R^3 = H$ (Lys),
6 : Gram negative PG: $R^1 = OH$, $R^2 = COOH$, $R^3 = H$ (DAP)

Figure 2. General repeating unit of peptidoglycan (PG) of Gram-positive (**5**) and Gram-negavtive bacteria (**6**).

The fact that compounds originating from bacterial PG can trigger the innate immune system encouraged the synthesis of well-defined PG fragments in order to determine the structural elements required for receptor activation. These synthetic efforts led to D-glutamyl-(2S,6R)-diaminopimelic acid (*i*E-DAP, **7**)[6] and muramyl dipeptide (MDP, **8**)[7,8] as minimal active structures for the NOD1 and NOD2 receptor, respectively (*Figure 3*).[1,9] Synthetic lipopeptide Pam_3CSK_4 (**9**) was found to be a ligand for TLR2.[10]



7 **8** **9**

Figure 3. NOD1 ligand *i*E-DAP (**7**), NOD2 ligand MDP (**8**) and TLR2 ligand Pam_3CSK_4 (**9**).

More fundamentally, well-defined PRR ligands with associated immunological activity are valuable tools to elucidate the mechanism of the immune system at a molecular level.[13,14] Although a lot of knowledge is acquired with compounds isolated from biological sources, the availability of synthetic compounds is indispensable. In comparison with biological isolated material, synthetic

compounds are normally easier to purify to homogeneity thereby lacking potential biologically active contaminations. In addition synthetically prepared compounds can be designed and prepared that allow the installation of suitable handles such as fluorescence labels.[15-17]
Structurally well-defined ligands of PRRs with either agonistic or antagonistic properties are equally interesting in biomedical science. Agonists can be implemented in the development of improved or new vaccines against infectious agents or tumors, while antagonists are relevant research targets to combat autoimmune diseases. Besides malfunctioning PRRs are involved in immunological disorders such as Crohn's disease, chronic inflammatory bowel disease and Blau syndrome.[11,12]

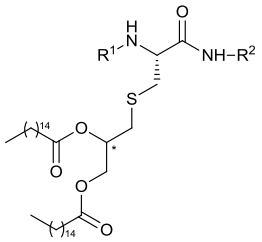
1.2 Structure activity relation of PRRs: TLR2, NOD1 and NOD2

Although a lot of attention has been devoted to the development of PRR ligands with a specific immunological profile, the number of structurally well-defined ligands known to date is relatively low. Major advances have been made with PRR agonists, of which TLR2 (Pam_3CSK_4), TLR4 (lipid A analogues), TLR7 (imiquimod) and TLR9 (CpG oligonucleotides) are relevant examples of synthetically accessible ligands. Similarly, attention has been directed to the synthesis and evaluation of PG fragments as ligands for TLR and NOD receptors.[1,14,18-27] In the next sections an overview is presented on the structure activity relation (SAR) and evaluation of ligands for TLR2 and NOD1 and NOD2.

1.2.1 TLR2 ligands

Lipoproteins present in the cell wall of Gram-positive and Gram-negative bacteria were the first natural TLR2 ligands isolated and recognized as TLR2 agonist. Diacylated Pam_2CSSNA (**10**) isolated from natural sources was found as the minimal structure to function as active TLR2-ligand (*Figure 4*).[28] SAR studies revealed that the length and nature of the peptide are important for recognition: Cys-Ser lipodipeptide was found to be an active combination with the shortest peptide length while the single dipeptide and the fatty acid were inactive.[29,30] Other relevant examples of synthetic diacylated ligands for TLR2 are MALP-2 (Pam_2GNNDESNISFKEK, **11**),[29,31] a *Mycoplasma fermentans* derived lipopeptide and FSL-1 (Pam_2CGDPKHPKSF, **12**),[31,32] a lipopeptide derived from *Mycoplasma salivarium*. TLR2 activation was also attained by lipoproteins originating from *Escherichia coli* and numerous other Gram-negative bacteria. These lipoproteins are provided with three fatty acid tails because of the presence of an additional

N-acylated fatty acid chain with respect to peptides above. SAR studies with synthetic triacylated derivatives led to the discovery of Pam_3CSK_4 (**9**) as an active TLR2 ligand with improved solubility.[10,33] Importantly, it was discovered that receptor dimerization was decisive for ligand recognition. The TLR2/6 dimer preferentially recognizes diacylated lipopeptides, such as MALP-2 (**11**) and FSL-1 (**12**), whereas the TLR1/2 dimer recognizes triacylated lipopeptides, like Pam_3CSK_4 (**9**).[31,34]



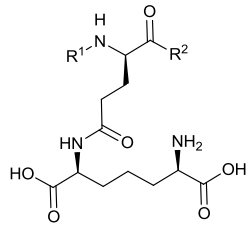
9 $R^1 = CO(CH_2)_{14}CH_3$, R^2 = SKKKK
10 R^1 = H, R^2 = SSNA
11 MALP-2 R^1 = H, R^2 = GNNDESNISFKEK
12 FSL-1 R^1 = H, R^2 = GDPKHPKSF

Figure 4. TLR2-L Pam_3CSK_4 (**9**), Pam_2CSSNA (**10**), FSL-1 (**11**) and MALP-2 (**12**).

With the objective to attain a TLR2 agonist with improved immunological profile, SAR studies were executed using Pam_3CSK_4 as lead compound. Pam_3Cys consists of a mixture of diastereoisomers because a racemic glycerol derivative was used for its synthesis. It was found by several groups that enantiopure Pam_3Cys derivatives containing the *R*-configurated glycerol are somewhat more potent immune-stimulating agents. Due to the synthetic efforts to obtain chirally pure Pam_3Cys and the commercial availability of Pam_3Cys as a mixture of diastereoisomers most studies are still based on diasteromeric mixtures.[35-37] To evaluate the influence of fatty acid chain length, Ulmer and co-workers varied the chain length of the fatty acids at the glycerol moiety and the fatty acid connected to the *N*-terminal position of the cysteine.[38,39] A minimum chain length of 8 carbons was required for the ester bound fatty acids on the glycerol moiety and increasing the number of carbons led to a rise in activity. Interestingly, differences between human and mouse TLR2 were found, and human TLR2 required longer chain-length for ligand recognition. In addition, more variation of the *N*-terminal fatty acid chain was allowed because this chain contributed only minimally to human TLR2 recognition. Furthermore, the influence of the cysteine in the Pam_3Cys ligand was subject of research.[40] In an extensive study a 95-member library of Pam_3Cys derivatives was constructed in which various proteinogenic amino acid combinations were evaluated. However, none of the obtained Pam_3Cys derivatives substantially exceed the biological activity of Pam_3CSK_4.[40]

1.2.2 NOD1 ligands

The best known members of the family of NLRs are NOD1 and NOD2. Both NOD1 and NOD2 recognize fragments of peptidoglycan (PG) of microbes. With the aid of chemically synthesized PG fragments (2S,6R)-*i*E-*meso*DAP (**7**) was found to be the structurally minimally active component for NOD1, while the analogue elongated with L-Ala (**13**) turned out to be more active (*Figure 5*).[41,42] Based on the biological processing of PG, it was hypothesized that **13** exhibits an improved uptake and therefore an increased potency. In another SAR study the *C*-terminal ends of tripeptide L-Ala-*i*E-DAP (**13**) and dipeptide *i*E-DAP (**7**) were amidated giving L-Ala-*i*Q-DAP (**14**) and *i*Q-DAP (**15**) respectively. This conversion into terminal amides resulted in the abolishment of activity indicating that the carboxylic acid is essential for binding to NOD1.[43]



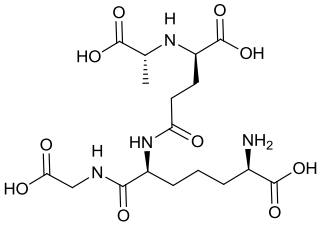
7 R^1 = H, R^2 = OH
13 R^1 = L-Ala, R^2 = OH
14 R^1 = L-Ala, R^2 = NH_2
15 R^1 = H, R^2 = NH_2

Figure 5. Modifications of *i*E-DAP (**7**): **13** – **15**.

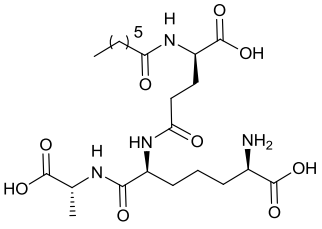
NOD1 ligands are relatively poor immune stimulatory agents and high concentrations are necessary to induce cytokines.[44] In the early 80s the Fujisawa Pharmaceutical group reported together with NOD1 ligand *i*E-DAP (**7**), ligand FK-156 and FK-565 (*Figure 6*) as a compound with improved immune-stimulating potency.[6,45] They further modified *i*E-DAP with steaoryl (**16**) or caprylyl (**17**) fatty acids to investigate the effect of lipophilic *i*E-DAP derivatives. The acylated *i*E-DAP derivatives were found to be as potent as FK-156. Synthetic efforts by the group of Fukase confirmed that appendage of lipophilicity by acylation resulted in ligands with enhanced immunogenic profile. Myristoyl (**18**), pentadecanoyl (**19**) and palmitoyl (**20**) *i*E-DAP derivatives are 100-fold more potent than the original *i*E-DAP.[46]

In bacteria such as *Mycobacterium* the most common stereoisomer of diaminopimelic acid is *meso*-(LD)DAP. LL-DAP is found in specific bacteria such as *Clostridium perfringes*, whereas DD-DAP has not been isolated so far. With the objective to assess the influence of the stereochemistry of diaminopimelic acid on NOD1 binding, Uehara *et al.* prepared and evaluated the individual LL-, DD- and meso- stereoisomers of DAP, starting from a commercially available

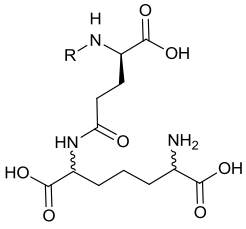
mixture.[44] *Meso*DAP was the most active isomer, whereas LL-DAP was less active and DD-DAP was inactive in most assays. This finding was confirmed by Hasegawa *et al.* who synthesized a library of acylated *i*E-DAP derivatives using a diasteromeric mixture of DAP.[47] David and co-workers also used a stereoisomeric mixture of DAP to create a library of C_{12}-γ-D-Glu-DAP (**18**) derivatives. Their structure activity relationship studies showed amongst others that replacement of glutamic acid by glutaric or γ-aminobutyric acid led to a reduced activity.[48] In addition, it was concluded that the carboxylic acids of DAP can only be converted into esters with maintenance of immunological activity.



FK156



FK565



16 R = $CO(CH_2)_{16}CH_3$,
17 R = $CO(CH_2)_6CH_3$,
18 R = $CO(CH_2)_{12}CH_3$
19 R = $CO(CH_2)_{13}CH_3$
20 R = $CO(CH_2)_{14}CH_3$

Figure 6. Compounds FK-156, FK-565 and **16** – **20**.

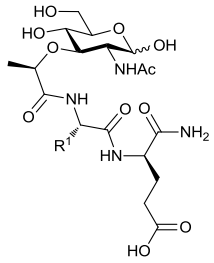
1.2.3 NOD2 ligands

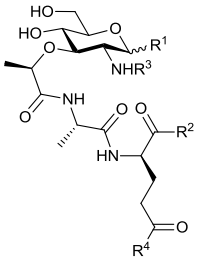
The NOD2 receptor is expressed intracellularly like the NOD1 receptor and recognizes similar peptidoglycan fragments.[7,49] Muramyl dipeptide (MDP, **8**) is the minimal structure that binds to the NOD2 receptor but the activity of MDP is low and high concentrations of this ligand are required. With the objective to obtain a more active NOD2 ligand with a minimum of side effects, extensive research to the synthesis and evaluation of MDP derivatives was carried out. Recently an overview on the structure activity relation of MDP derivatives was published.[50]

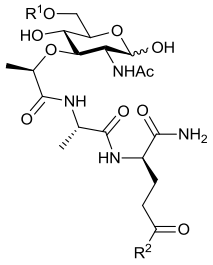
MDP (**8**) consists of muramic acid (MurNAc), the lactic acid functionalized glucosamine, linked to a dipeptide tail of which alanine has the L-configuration and isoglutamine the D-configuration (*Figure 7*). The biological activity of MDP is lost when the configuration of these amino acids is changed, especially isoglutamine.[7,51] The nature of the amino acids in the dipeptide of MDP derivatives proved to be less decisive because alanine (**8**) could be replaced by serine (**22**), valine (**23**) or proline (**24**).[52] Isoglutamine (**8**) in turn can be substituted by glutamic acid (**25**) with retention of activity. PG muropeptides of bacteria may differ and a study using PG of *Staphylococcus aureus* confirmed

that the glutamic acid modification does not significantly influence the immunostimulatory capacity.[53]

Modifications of the MurNAc moiety in MDP were also evaluated. Examples of modifications of the anomeric position of MurNAc are *S*-glycosides (**26**), and *O*-aryl/ *S*-aryl analogues (**27**).[54] Fatty acids were also installed at the anomeric center, showing that the nature of the aglycon in MDP contributes to the activity. Further it was found that the β-anomers are more active.[50] Replacement of the *N*-acetyl at the C-2 position of MurNAc by an *N*-glycolyl (**28**) led to an increased activity.[55] In contrast to these results replacement of the amino function in MDP was detrimental to biological activity.[56] Modifications at the C-4 and C-6 positions turned out to be appropriate to enhance the activity. Uehori *et al.* synthesized several C-4 and/or C-6 acyl-modified MDP derivatives: single C-6-octanoyl (**29**) or C-6-stearyl (**30**) fatty acid modifications enhance the ligand activity. In addition, it was shown that these ligands act as TLR2 and TLR4 inducers in human DCs.[57] Other groups used the C-6-position in MurNAc to obtain biologically active probes such as biotinylated MDP (**31**) to study ligand-receptor binding.[58,59] The favorable influence of lipophilic tails on the activity of MDP resulted in various derivatives of which B30-MDP (**32**)[60] and Murabutide (**33**)[50] are the most well known. Today these derivatives are still used in several therapies because of their reduced side effects and good immunogenicity.[61]







8 $R^1 = CH_3$
22 $R^1 = CH_2OH$
23 $R^1 = CH_2(CH_3)_2$
24 $R^1 = (CH_2)_3$

25 $R^1 =$ $R^2 = OH$, $R^3 = Ac$, $R^4 = NH_2$
26 $R^1 =$ SH, $R^2 = NH_2$, $R^3 = Ac$, $R^4 = OH$
27 $R^1 =$ O-Aryl or S-aryl, $R^2 = NH_2$, $R^3 = Ac$, $R^4 = OH$
28 $R^1 = OH$, $R^2 = NH_2$, $R^3 = COCH_2OH$, $R^4 = OH$

29 R^1 = octanoyl, $R^2 = OH$,
30 R^1 = stearoyl, $R^2 = OH$,
31 R^1 = biotin, $R^2 = OH$,
32 R^1 = mycoloyl, $R^2 = OH$,
33 $R^1 = H$, R^2 = Lys(stearoyl)

Figure 7. Modifications on MDP (8): 22 – 33.

1.3 Interaction between PRRs

A lot of progress has been made on the elucidation of the signaling pathways of the innate immune system upon binding of PAMPs to PRRs. The broad structural variety of PAMPs and the expression of several types of PRRs by immune cells suggest that interaction of their signaling pathways occurs. This mutual interaction of TLRs may mean that a specific PRR influences the expression of a

different PRR. Interaction of PRRs may also lead to alteration of their ligand specificity. Besides, the cytokine production, a PRR can synergize with or oppose the responses of other PRRs.[62,63] For instance MDP and LPS or lipoteichoic acids exhibit a synergistic effect with respect to the IL-8 production in human monocytic cells in culture.[64] Although focusing on different research objectives, a lot of studies describe synergism of MDP or muropeptides and LPS when measuring the production of cytokines such as TNFα, IL-6, IL-8 and IL-10 with human primary cells.[51,65-67] Boons *et al.* synthesized and evaluated several fragments of muramyl tripeptide (MTP), containing lysine (MTP-Lys) or diamino pimelic acid (MTP-DAP) moieties. They discovered that not only MDP but also MTP-DAP and MTP-Lys are synergistic with LPS.[23,43,66] This effect was further investigated by Takada and co-workers, using NOD1 ligand FK-156 in combination with synthetic TLR4 ligand Lipid A, TLR2 ligand Pam_3CSSNA and TLR9 ligand CpG. All ligand combinations showed a synergistic effect, considering the IL-8 production of human monocytic cells. [63,68] Interestingly, Tada *et al.* reported that TLR2-ligand Pam_3CSSNA with MDP or FK565 did not induce synergistic generation of IL-12 in human DCs while combination of these NOD1 and NOD2 ligands with lipid A and CpG resulted in synergism.[69] With the objective to elucidate the immunological processes underlying Crohn's disease, Watanabe and co-worker reported that MDP is a negative regulator of a TLR2 receptor mediated response.[70]

The interaction between PRRs is a complex process, the study of which is still in its infancy. In this respect, more insight in issues such as ligand processing and ligand recognition is necessary. For instance, it is well established that lipopeptides function as ligands for TLR2 located at the cell membrane and PG fragments as ligands for cytosolic NOD receptors. On the other hand the role of PG as ligand for TLR2 is controversial. In some studies PG is described as TLR2 ligand[22,71-73] while others report that MDP and PG are not recognized by TLR2[49,74] or that they activate both the receptors.[17]

1.4 PRR-ligand based conjugates

Vaccination is one of the most important achievements of modern medicine. Classical vaccines have their limitations, such as undesired side effects and difficulties to target specific infectious diseases, such as influenza. The prospect to develop immunotherapy for the treatment of cancer further stimulates the search for new types of vaccines. Advances in understanding of immunological processes, such as the interaction of the innate and adaptive immune system is an incentive to explore new vaccines having a well defined molecular composition and corresponding immunological properties.[75] A valuable approach to reach this goal is represented by the design, synthesis and evaluation of

conjugates consisting of structurally defined and covalently bound PRR ligands and specific epitopes. In the following section selected examples of these conjugates are discussed.

1.4.1 TLR2 based conjugates

In 1989 Deres *et al.* reported as one of the first that a synthetic conjugate, in which a TLR2 ligand is covalently linked to a peptide epitope, exhibits an improved immunogenic response. They synthesized conjugate **34** (*Figure 8*), consisting of an antigenic peptide derived from influenza virus and the TLR2 ligand tripalmitoyl-*S*-glycerylcysteinyl-seryl-serine (Pam_3CSS) and showed that this conjugate was able to induce efficient priming of influenza-virus-specific cytotoxic T lymphocytes (CTL) *in vivo*.[76] A few years later several other groups reported that these favorable properties were also valid for conjugates of TLR2 ligands with other antigenic peptides.[77-80] Based on the increased immunostimulating properties of epitopes in the form of dendrimers, Zeng *et al.* prepared and evaluated tetrameric polyoxime constructs (**35**) in which a model antigen derived from *influenza hemaglutinin*as and Pam_3Cys are incorporated.[81] The TLR2 ligand in **35** proved to be essential to induce significant peptide-specific antibody responses. Jackson and co-workers broadened the scope of TLR2 conjugates by the incorporation of two epitopes. The new conjugates (**36**) consist of a T helper epitope, a target epitope and TLR2 ligand Pam_2Cys.[82] The selected target epitopes are either recognized by $CD8^+$ T cells, such as epitopes from influenza virus, the bacterium *Listeria monocytogenes* and ovalbumin as a model tumor antigen, or recognized by B cells, such as epitopes from luteinizing hormone releasing hormone (LHRH) and the hormone gastrin. In mouse models it was shown that the conjugates were capable of inducing either $CD8^+$ T cell or antibody-mediated immune responses.

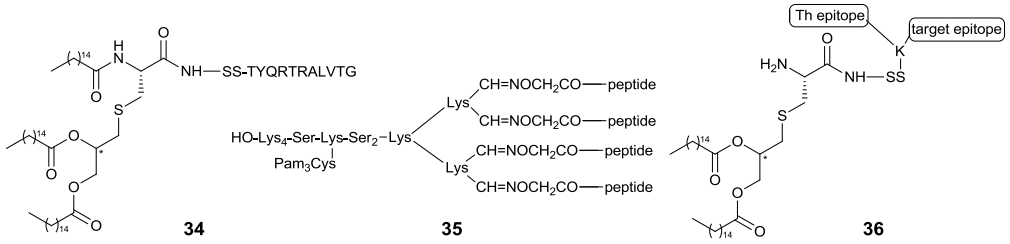
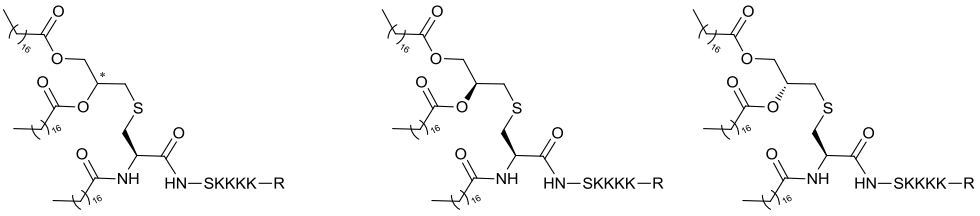


Figure 8. TLR2 ligand based conjugates **34** – **36**.

In a collaborative research project between the bio-organic synthesis group and the tumor-immunology group of Leiden University, TLR2 conjugates, comprising

racemic Pam_3CSK_4 (**37** – **40**) or chiral pure Pam_3CSK_4 (**41** and **42**) and OVA peptides, were prepared and immunologically evaluated (*Figure 9*).[37,83] The epimeric mixtures (**37** – **40**) induced DC maturation to the same extent as the free racemic TLR2 ligand and enhanced *in vitro* antigen presentation in comparison with mixtures of the free ligand and epitope. The conjugates displayed both *in vitro* and *in vivo* an enhanced uptake. Interestingly, with the aid of fluorescent conjugates **39** and **40** and a TLR2 deficient cell line it was shown that uptake of the conjugates was independent of TLR2 expression. In a subsequent study to determine the influence of the chiral centre at C-2 of the glycerol moiety in Pam_3CSK_4 it was shown that *R*-epimer **42** is the more potent stereoisomer. Notably, the epimeric mixture is as potent as the *R*-epimer in DC maturation and antigen presentation.



37 R = DEVSGLEQLESIINFEKL
38 R = DEVSGLEQLESIINFEKLAAAAAK
39 R = C(Alexa-488)DEVSGLEQLESIINFEKL
40 R = C(Bodipy FL)DEVSGLEQLESIINFEKL

41 R = DEVSGLEQLESIINFEKL **42** R = DEVSGLEQLESIINFEKL

Figure 9. TLR2 ligand based conjugates **37** – **42**.

Apart from conjugates consisting of a peptide epitope and a TLR ligand a lot of attention is given to conjugates in which the epitope is represented by a carbohydrate structure. A main incentive for this research is the finding that a lot of tumors are characterized by the presence of uniquely or excessively expressed glycans at their cell surface. These tumor-associated carbohydrate antigens are interesting targets for the development of therapeutic cancer vaccines or immunotherapies.[84] However, usually carbohydrates are poorly immunogenic and induce T cell-independent immune responses. Conjugation of a carbohydrate epitope to a carrier protein can enhance the presentation of carbohydrate antigens and induce helper T cell activation. Insight in the role of PRRs for immune responses led to the development of fully synthetic conjugates in which TLR ligands are incorporated. Important examples of this type of conjugates are reported by the group of Boons. In 2005 they reported the synthesis and evaluation of conjugate **43**, comprising the tumor-associated MUC-1 glycopeptide B-cell epitope, the universal helper T epitope YAFKYARHANVGRNAFELFL (YAF), and the lipopeptide (Pam_3CSK_4).[85] In an extension of this study, structure activity studies were performed leading to

conjugate **44**, comprising TLR2-L Pam_3CSK_4 combined with a helper T-cell epitope from polio virus and in comparison with **43** an elongated MUC1 B-cell epitope.[86,87] It was concluded that three-component conjugates can elicit exceptionally high titers of IgG antibodies that recognize the cancer cells expressing tumor-associated carbohydrate in mice. For the synthesis of these conjugates carbohydrate, peptide and lipid chemistries had to be combined. A difficulty is represented by the acidic conditions used for the cleavage of oligopeptides from a resin in solid phase peptide synthesis that are not always compatible with the repeating acetal functions in oligosaccharides. The Boons group developed a convergent route of synthesis in which the oligopeptide was separately synthesized with the aid of a solid phase synthesis approach and condensed off-resin with a protected carbohydrate based antigen derivative.
The group of Kunz, having a track record on the synthesis of glycopeptides, assembled a number of conjugates in which TLR2-L Pam_3CSK_4 was linked *via* an ethylene glycol spacer to a variety of MUC1 glycopeptide antigens (**45** – **47**) (*Figure 10*).[88] The glycopeptides were assembled via solid phase peptide synthesis and were linked off-resin to separately prepared Pam_3CSK_4, functionalized with the glycol spacer, to give conjugates **45** – **47**. All these conjugates elicited immune responses in mice, although not as high as those for the corresponding MUC1 tetanus toxoid vaccine. With the aid of the azide-alkyne Huisgen copper-catalyzed click reaction mono-, di- and tetravalent MUC1 glycopeptides, linked to one TLR2 ligand Pam_3CSK_4 were prepared (**48** - **50**) (*Figure 10*).[89] The oligovalent conjugates proved to have more immunostimulatory potency than the corresponding monovalent conjugates. Renaudet and co-workers prepared an advanced conjugate in which a cyclic peptide functions as a delivery system for a TLR2 ligand, OVA $CD8^+$ CTL epitope, T helper epitope and a cluster of tumor associated carbohydrate B-cell epitopes (**51**) (*Figure 11*). The constructs induced antitumor B and T cell protective immunity.[90]

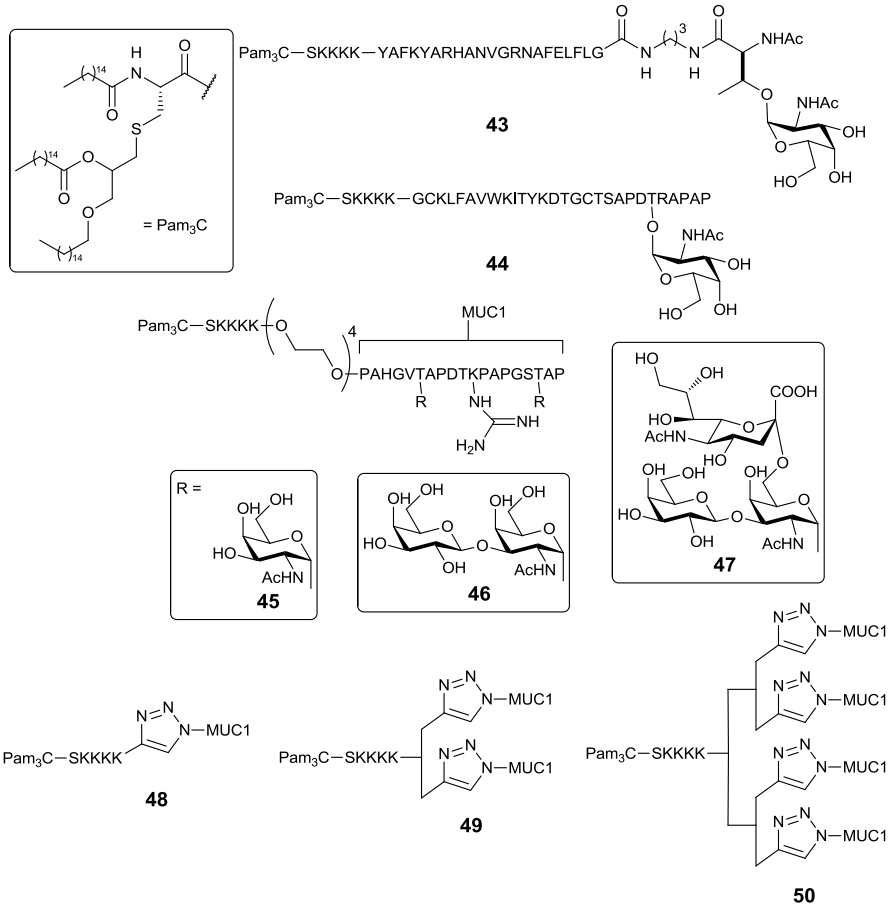


Figure 10. TLR2 ligand based carbohydrate conjugates **43** –**50**.

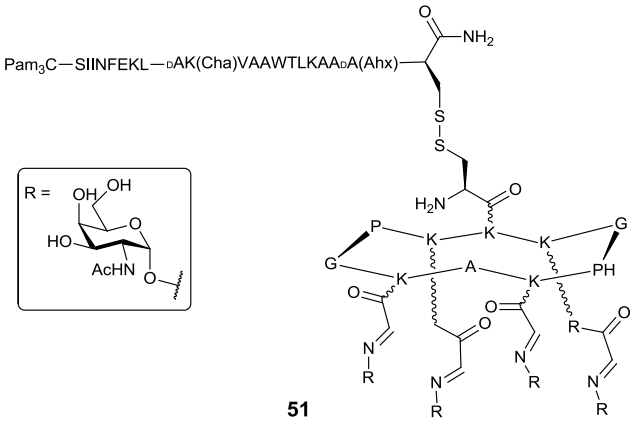
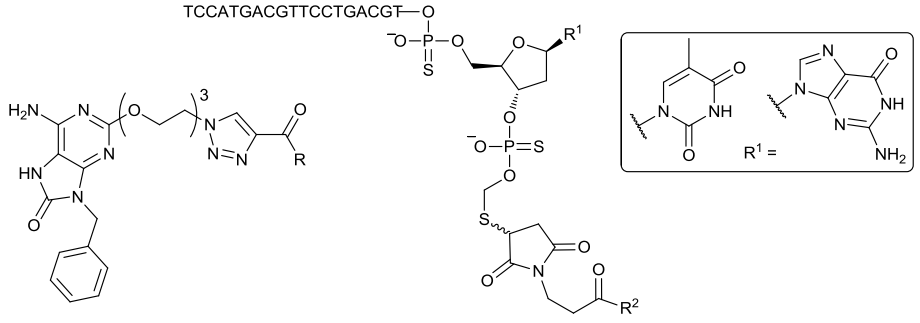


Figure 11. TLR2 ligand based carbohydrate conjugates **51**.

1.4.2 Other TLR based conjugates: TLR7 and TLR9

Conjugates that contain other TLR ligands have also been explored. For instance, Khan and Weterings prepared and evaluated TLR7 ligand-conjugates (**52**, **53**) bearing short or long versions of the well-known OVA-derived $CD8^+$ T-cell epitope SIINFEKL (*Figure 12*). Although these conjugates did not show DC activation, the antigen presentation was still intact with respect to the free peptides.[91] The position and the nature of the linkage of the TLR ligand to the peptide epitope is important for activity. Wu and co-workers showed that 8-oxoadenine derivatives, differently conjugated to murine serum albumin were able to release cytokines *in vitro*.[92]



52 R = DEVSGLEQLES**II**NFEKL
53 R = DEVSGLEQLES**II**NFEKLAAAAAK

54, **55** R^2 = DEVSGLEQLES**II**NFEKL
56, **57** R^2 = DEVSGLEQLES**II**NFEKLAAAAAK

Figure 12. TLR7 and TLR 9 conjugates

Additionally, Khan *et al*. reported an extensive study on the synthesis and evaluation of TLR 9 CpG conjugated peptides (**54 – 57**) (*Figure 12*).[83] The CpG conjugates and the corresponding TLR2 conjugates (**37**, **38**), as described above, follow a similar intracellular processing pathway that leads to a comparable level of antigen presentation and T-cell priming. The uptake of both types of conjugates proved to be TLR independent although the exact internalization routes of these conjugates differ.

1.4.3 NOD2 based conjugates

Relatively few examples are reported on the synthesis and evaluation of conjugates in which NOD ligands are incorporated. Li and co-workers prepared different conjugates of the anti-tumor drug Paclitaxel (Taxol®) and NOD2 ligand MDP.[93] Conjugate 2'-*O*-MTC-01 (**58**) did not only induce antitumor immunity but also showed immune-enhancing effects by improved production and expression

of TNFα and IL-12 in comparison with single Paclitaxel and MDP in mouse models.

Already in 1982 Carelli and co-workers showed that immunological neutralization of the hypothalamic luteinizing hormone-releasing hormone (LHRH) was achieved by a conjugate consisting of a decapeptide fragment of the hormone and MDP.[94] In 2001, the same group showed that immunization with a conjugate consisting of an oligopeptide, derived from a growth hormone and ovalbumin, and MDP exhibits long-lasting rat growth enhancement.[95] The group of Wardowska studied conjugates in which MDP and the phagocytosis stimulating tetrapeptide tuftsin are combined. Several combinations such as tuftsin (TKPR) (**59**) and retro-tuftsin (RPKT) (**60**) were investigated (*Figure 13*). The MDP-(retro)tuftsin conjugate proved to have the most beneficial biological activities in comparison with the single compounds.[52,96,97]

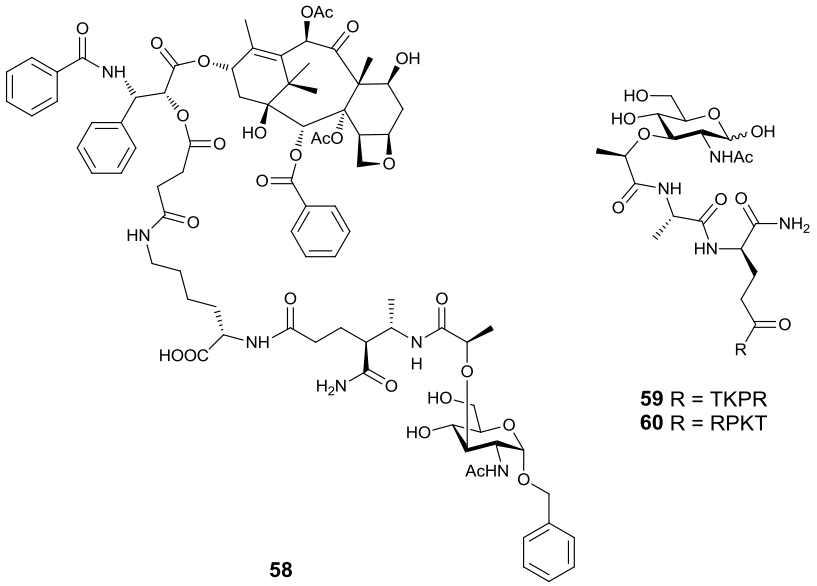


Figure 13. NOD2 based conjugates **58** – **60**.

Despite the enormous progress made over the last decades, there still is much research needed to obtain prophylactic and therapeutic vaccines for several diseases. In this framework much is expected from conjugates, such as described above to elucidate immunological processes at a molecular level. Newly designed conjugates comprising other structurally defined PRR ligands, other epitopes or combinations thereof may lead to synthetic structurally defined vaccines.

1.5 Aim and outline of this Thesis

The research described in this Thesis aims at the improvement of the immunological profile of NOD1, NOD2 and TLR2 ligands and conjugates with one or two of these PRR ligands covalently bound to peptide epitopes. In the introductory chapter an overview is presented on known ligands of NOD1, NOD2 and TLR2. Selected examples are given of conjugates in which these ligands are incorporated.

Chapter 2 describes the synthesis and immunological evaluation of four NOD2 ligand-antigen conjugates. In these conjugates the NOD2 ligand MDP is covalently bound to an antigenic peptide at its *C*- or *N*-terminus *via* two different positions in the MDP moiety, namely the anomeric center or the isoglutamic acid moiety. The outcome of the immunological evaluation of these conjugates was disappointing. To enhance the immunostimulatory properties of the NOD2-L-antigen conjugates the synthesis of lipophilic NOD2-L derivatives was undertaken.

Chapter 3 describes the synthesis and evaluation of a set of three modified NOD2 ligands in which a fatty acid is appended at different positions in the MDP derivative. After immunological evaluation the most potent ligand was conjugated to an antigenic peptide. The immunological profile of this conjugate was satisfactory but not better than a comparable TLR2 conjugate.

Chapter 4 describes the preparation of eight NOD2-L/TLR2-L bis-conjugates. The set of conjugates differs in ligand position (*C* and/or *N*-terminal) and method of conjugation. All conjugates were found active in the NOD2 and TLR2 specific assays and were able to produce the desired cytokines. So far an additive or synergistic effect by the PRR ligands in the immunological assessment was not found.

Chapter 5 evaluates the potency of NOD1-ligand based conjugates and describes the synthesis and immunological probing of two NOD1 ligands, two NOD1-L-antigen conjugates and two NOD1/TLR2-L bis-conjugates. The conjugation of the designed NOD1 ligand led to a complete abolishment of NOD1 recognition and marginal immunostimulatory potency. In contrary, the single ligands *i*E-DAP and a C12-derivative were recognized, though showed a low potency.

Chapter 6 describes the synthesis and immunological evaluation of urea-derived TLR2-ligand derivatives. This so-called $Upam_3CSK_4$ turned out to be more potent than TLR2 ligand Pam_3CSK_4. The conjugation of this urea derived ligand with the antigenic peptide led to a construct with a slightly improved DC maturation and an antigen presentation similar to the established TLR2-conjugate. A library is synthesized that comprises the modification of the serine position by eighteen different (non)-natural amino acids to study the activity relation on this position. The substitution of serine with 2-aminobutanoic acid, allylglycine, propargylglycine or diaminobutyric acid proved to be the most interesting modifications for additional studies.

Finally, the research presented in this Thesis and some future prospects are summarized in **Chapter 7**.

The immunological evaluation of the synthesized compounds, described in this Thesis, has been carried out by F.A. Ossendorp, S. Khan and G.G.P. Zom of the Tumorimmunology Group, department of Immunohematology and Bloodtransfusion, from the Leiden University Medical Center.

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