Design, synthesis, and evaluation of antigenic peptide conjugates containing Toll-like receptor agonists

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Chapter 5

Synthesis and evaluation of Ovalbumin derived peptides functionalized with a TLR2 and TLR7 agonist

Introduction

Pattern recognition receptors (PRRs) belong to the first line of defense of vertebrates for the identification of pathogen invasions. In particular, PRRs are expressed by cells of the innate immune system such as antigen presenting cells (APCs) and each type of receptor has been evolved to detect specific highly conserved molecular moieties, that are termed pathogen associated molecular patterns (PAMPs) or damage associated molecular patterns (DAMPs). When these molecular patterns bind to the associated PRR on an APC many events are triggered, that initiate the host defense reactions and ultimately may result in an adaptive immune response. Several classes of PRRs are known such as, Nod-like receptors (NLRs), C-type lectin receptors (CLRs) and Toll-like receptors (TLRs). With the objective to obtain structurally well-defined adjuvants and ultimately vaccines, a lot of research is devoted to the development of synthetic accessible ligands for PRRs, of which the TLRs are most pursued. The human TLR family consists of ten members, each recognizing specific PAMPs.[1] TLRs are transmembrane proteins whose structure consists out of a leucine rich region that harbors the PAMP binding pocket, a transmembrane domain and a cytoplasmic tail called the TIL domain. Upon association with a PAMP, a TLR forms either a homo- or a heterodimer, which drives conformational adjustments in both receptors bringing both TIL-

domains together. This activates intracellular signalling cascades, leading amongst other to, cell-maturation, up-regulation of the cell- surface expression of co-stimulatory (such as CD80 and CD86) molecules and the induction of the expression of cytokines, (such as IL-12). TLRs are situated at different cellular locations, while TLR1, 2, 4, 5, and 6 occur at the cell surface, TLR3, 7, 8, and 9 reside in intracellular endosomal compartments. Synthetic ligands of relatively low molecular weight have been discovered for the TLR1/2 and TLR 2/6 combinations, for TLR4, and either selective or not for TLR7 and TLR8. Furthermore, structure-activity studies have culminated in several active agonists with the TLR4 ligand mono-phosphoryl lipid A (MPLA) as the first approved agonist for application as adjuvant in vaccines. Although much can be expected from the immunological properties of individual TLRs, multiple PRRs are activated upon a pathogen attack, making it an incentive to investigate the potential synergistic effect of combinations of synthetically accessible PRR ligands. Many data have been obtained with the immunological evaluation of mixtures of separate PAMPs.[2-4] Apart from this, the approach of the group of Esser-Kahn stands out, who show the great potential of covalently linked bi- and tri-agonists.[5-7] The design of such multi-agonists is challenging as their activity depends on the nature of the composing PAMPS, the position of attachment and the linker that interconnects them.

In line with the possible synergistic activity of covalently connected PAMPS[5-11] is the evolvement of the completely synthetic vaccines in which one structurally defined TLR or other PRR ligand has been covalently fused with a synthetic oligopeptide, containing a specific epitope. The favorable immunological properties of these vaccine modalities have been demonstrated many times. Synthetic peptides, containing epitopes of different origin have mostly been covalently connected to TLR2 ligands.[12-30] However, other PRR ligands[31] have also been incorporated into conjugates with various antigens such as a TLR4 (Lipid A analogue[32-33]), a TLR7/8 (2-alkoxy-8-oxo-adenine,[34-36] 6-(4-amino-2-butyl-imidazoquinolyl)-norleucine[37]), a TLR9 (CpG oligonucleotide[12]) and a NOD ligand (muramyl dipeptide[17]). With the expectation that peptide conjugates incorporating more than one PRR ligand may exhibit an improved immunological profile, some of these types of conjugates have been designed, synthesized and immunologically evaluated, such as conjugates incorporating ligands for both NOD2 and TLR2 together with a class-I OVA epitope,[20] a conjugate harbouring ligands for the mannose-6-phosphate receptor and TLR7 together with a class-I OVA epitope[36] and conjugates having a combination of DC-SIGN ligands, a TLR7 agonist and a gp100 antigen.[38] This chapter describes the synthesis, and immunological evaluation of conjugates, in which the TLR7 ligand [4-((6-amino-2-butoxy-8-oxo-7,8-dihydro-9H-purin-9-yl)methyl)benzoic acid] and the TLR2 ligand [Pam_3CSK_4] are covalently attached to ovalbumin derived peptides, provided with $DEVA_5K$ (**1a**, **2a**) and HAAHA (**1b**, **2b**) as model peptides, containing an MHC I or MHC II epitope respectively (Figure 1). From now on in this chapter, all $DEVA_5K$ or HAAHA containing constructs will be referred to with an **a** or **b** affix, respectively.

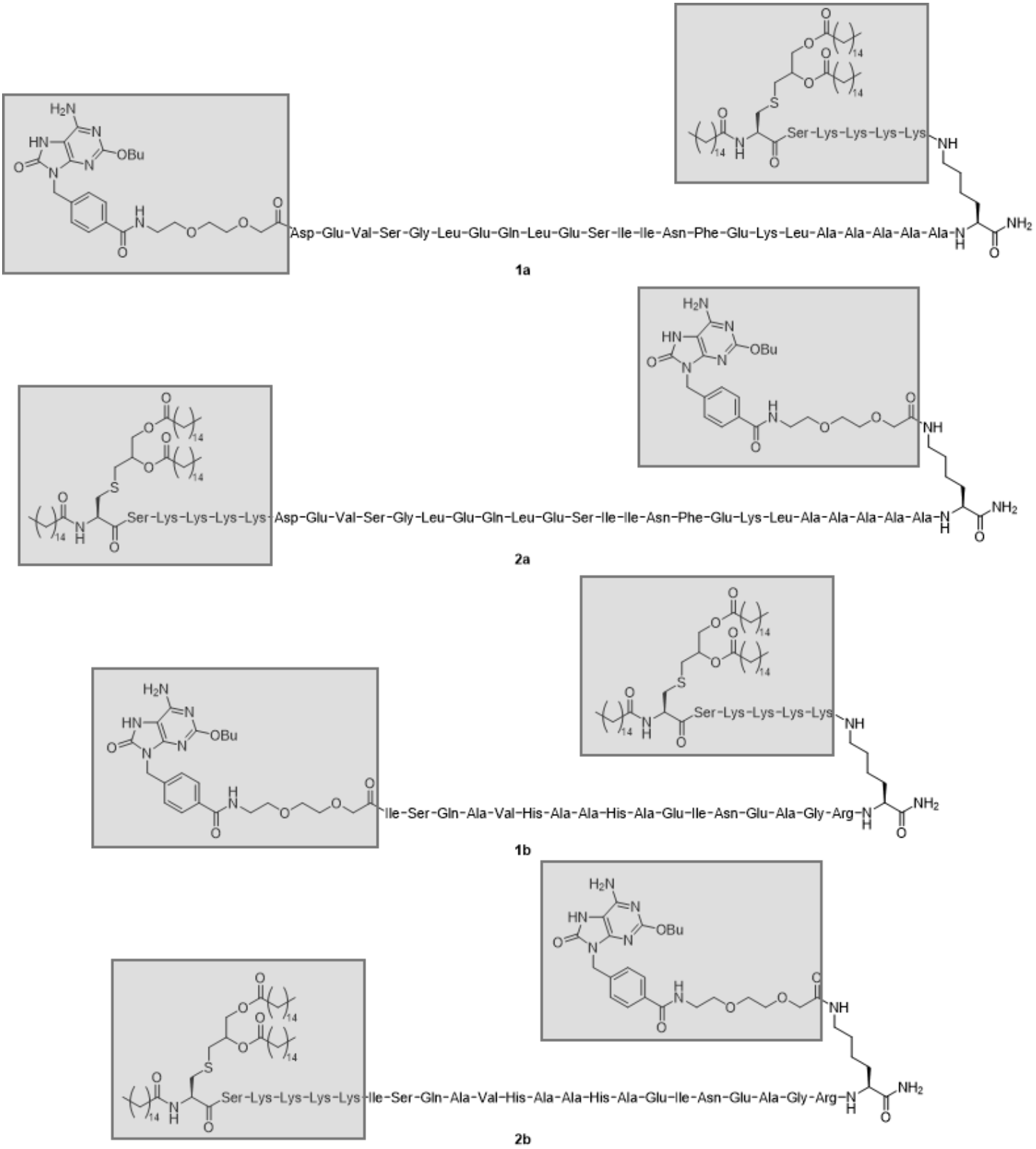
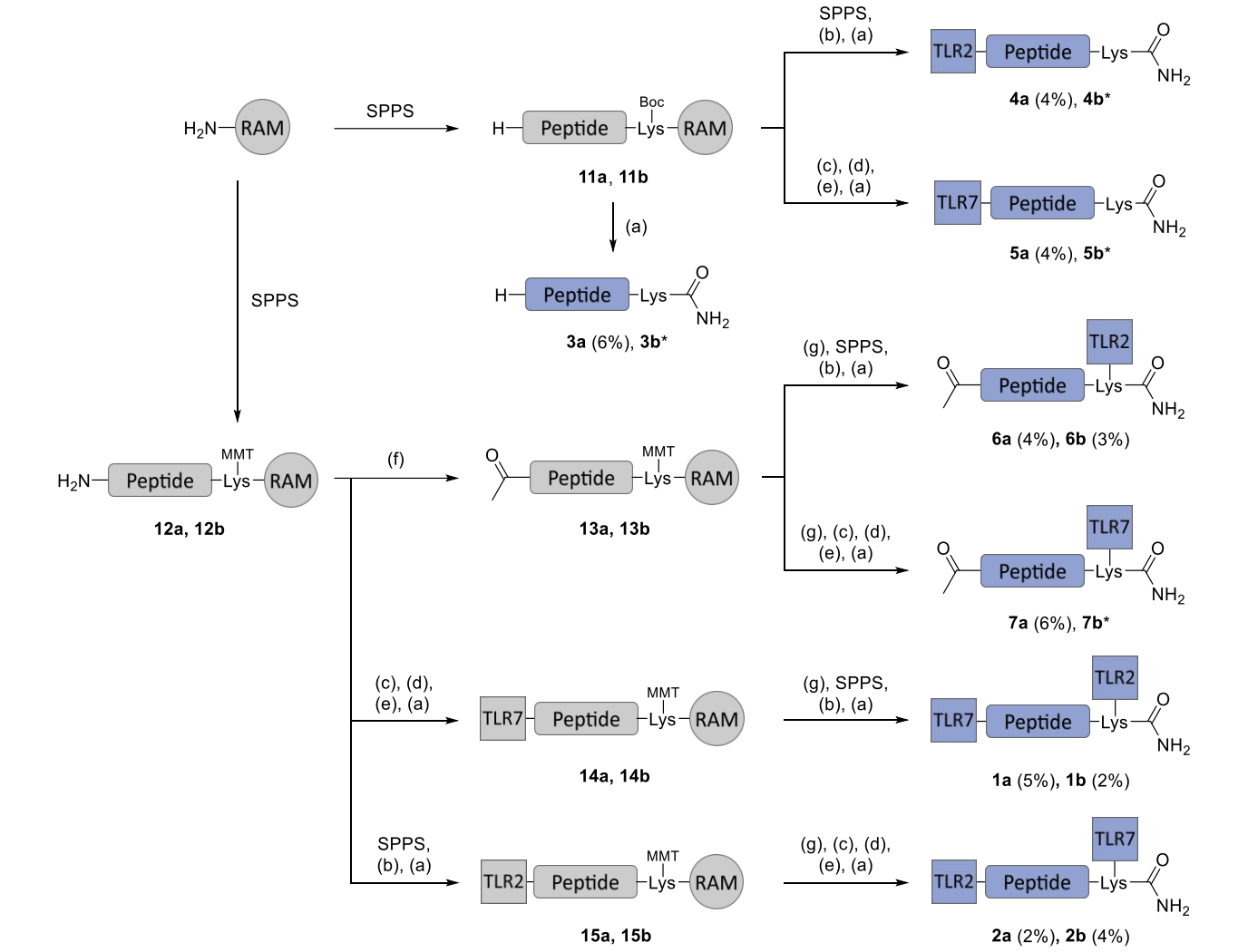


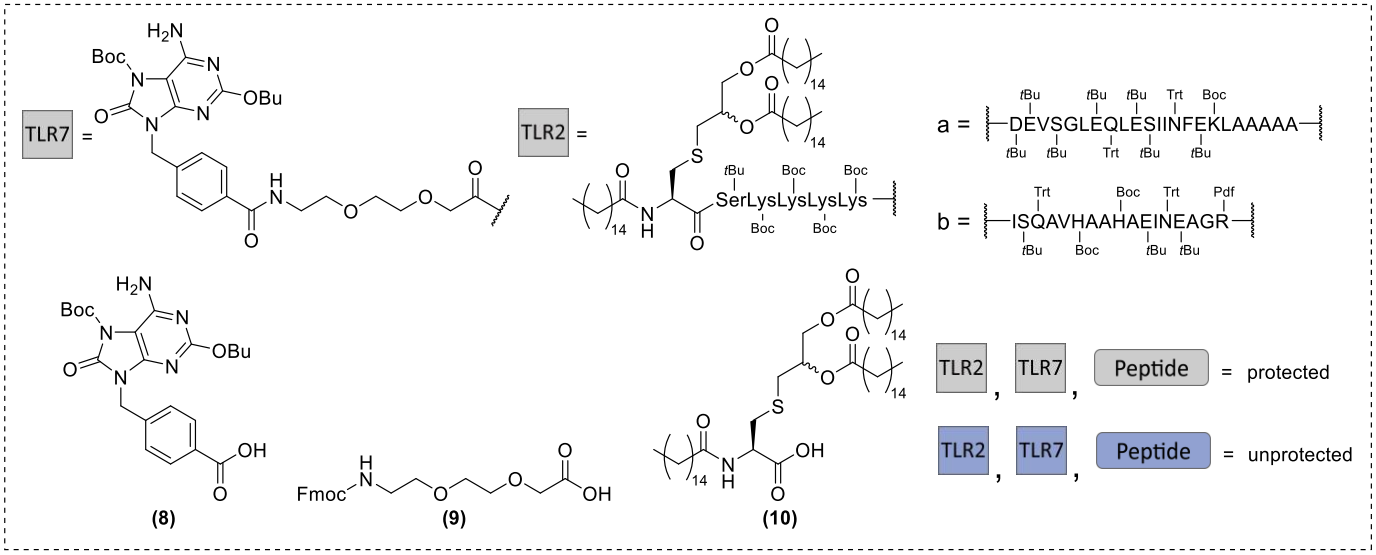
Figure 1: Structures of the target TLR2/TLR7 dual-functionalized ovalbumin antigen conjugates, where the individual structures of the ligands, including the connecting spacer are marked with a grey box. Conjugates **1a** and **2a** contain the $DEVA_5K$ sequence, and conjugates **1b** and **2b** contain the HAAHA sequence.

Results and Discussion

As shown in Figure 1, conjugate **1a** comprises an ovalbumin peptide, provided with MHC class I $DEVA_5K$ epitope that is connected at its N-terminal end to the TLR7 ligand, 4-((6-amino-2-butoxy-8-oxo-7,8-dihydro-9H-purin-9-yl)methyl)benzoic acid via the 8-amino-3,6-dioxaoctanoic acid spacer while the side chain of its C-terminal lysine is linked to the TLR2 ligand Pam_3CSK_4 via the Lys_4 spacer. Conjugate **2a** comprises the same constituents as **1a** while the TLR7 ligand and the TLR2 ligand have switched position. Conjugate **1b** corresponds with **1a** and conjugate **2b** corresponds with **2a** but in both cases the $DEVA_5K$ (**1a, 2a**) is replaced by HAAHA (**1b, 2b**) epitope. For the immunological evaluation of these target dual conjugates the following control compounds are needed; (i) the individual peptides **3a**, **b** (ii) the corresponding single functionalized conjugates linked at the N-terminal end with either a TLR2 **4a**, **b** or TLR7 ligand **5a**, **b** (iii) the corresponding single functionalized conjugates linked at the C-terminal end with either a TLR2 **6a, b** or TLR7 ligand **7a**, **b** (see Scheme 1).

All compounds were assembled using a TRIBUTE® Peptide Synthesizer and Tentagel S RAM as solid support, with the aid of a Fmoc chemistry elongation protocol, in which all amino acids were coupled twice under influence of HCTU as condensing agent. The thus automatically produced immobilized peptides were subsequently manually functionalized at the selected N- or C- ends with either one (TLR2L and TLR7L) or with consecutively both ligands (Scheme 1). The synthesis of the protected TLR7 ligand (**8**) is described elsewhere,[35] whereas spacer (**9**), TLR 2 ligand (**10**) and all amino acids are commercially available. Peptide **3b** and conjugates **4b**, **5b** and **7b** were synthesized by Niels Reintjes as described in his thesis.[39] Since the unfunctionalized peptide **3a** and the N-functionalized conjugates (either with a TLR2 **4a** or TLR7 ligand **5a**) do not require an orthogonal protecting group at the side chain of the C-terminal lysine, only standard amino acid building blocks were used for the SPPS of the immobilized precursor peptide **11a** (Scheme 1). Direct deprotection using TFA:TIS:H_2O and precipitation with a mixture 1:1 Et_2O:pentane was followed by centrifugation to give a pellet of the crude peptide. Isolation of the target peptides was attained by dissolving the crude material in 1:1:1 HO*t*Bu:H_2O:MeCN and subsequent RP-HPLC purification with a C18 column, providing $DEVA_5K$ peptide **3a** in 6% yield. The assembly of mono-conjugate **4a** with a N-terminal TLR2 ligand entailed the installation of the Pam_3SK_4 ligand at the N-terminal end of **11a**. Elongation of immobilized peptide **11a** with the spacer SK_4 was followed by the HCTU mediated condensation of palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-OH **10** to give the immobilized precursors of mono-conjugate **4a**. Similar to individual peptide **3a** all protecting groups were removed with concomitant release from the resin, precipitation and centrifugation to give the crude conjugate **4a** as a pellet. Finally, purification by HPLC, using a diphenyl column, yielded Pam_3CSK_4 functionalized $DEVA_5K$ conjugate **4a** in an overall yield of 4%. The assembly of the monoconjugate **5a** with a N-terminal TLR7 ligand started with applying the linker by condensation of immobilized and protected peptide **11a** with the commercially available 8-(Fmoc-amino)-3,6-dioxaoctanoic acid (**9**) under influence of HCTU. After the removal of the Fmoc group by treating the obtained resin three times with 20%





Scheme 1: Assembly of reference compounds **3a-7a**, **6b-7b** and dual conjugates **1a**, **1b**, **2a**, and **2b**. Below the scheme the structure of both TLR ligands and peptide sequences are given. Reaction Conditions: (a) TFA:TIS:H_2O 95:2.5:2.5, RT, 105 min; (b) Compound **10**, HCTU, DiPEA, 1:1 DCM:NMP, overnight; (c) Compound **9**, HCTU, D*i*PEA, DMF, overnight; (d) 20% piperidine in DMF, 3x 5 min; (e) compound **8**, HCTU, D*i*PEA, DMF, overnight; (f) Ac_2O, NMM, DMF, 5 min; (g) 1% TFA in DCM, 10x 30 sec then 2x 5 min.
*Peptide **3b** and conjugates **4b**, **5b** and **7b** were synthesized by Niels Reintjes.[39]

piperidine in DMF for 5 minutes, the released amine was coupled with TLR7 building block **8** using HCTU as condensing agent overnight to give the protected and immobilized progenitor of mono-conjugate **5a**. Removal of the protecting groups, cleavage from the solid support and HPLC purification, according to a similar procedure as described for **4a** resulted in the isolation of N-terminal TLR7 functionalized conjugate **5a** in 4% yield.

To obtain C-terminal mono- and dual functionalized conjugates, immobilized peptides (**12a, b**), containing the orthogonal MMT protecting group at the side chain of the C-terminal lysine were assembled using the same SPPS protocol (Scheme 1). En route to monofunctionalized TLR2 conjugates (**6a, b**) and TLR7 conjugate (**7a**), the SPPS of **12a, b** was followed by acetylation of the N-terminal amine with Ac_2O and NMM to give the capped peptide **13a, b**. Selective removal of the MMT group with 1% TFA in DCM, allowed for the introduction of each TLR ligand on the C-terminal end, using the same sequence of reactions and conditions, as described above for the appendage of TLR2 and TLR7 ligands at the N-terminal end (i.e. **4a** and **5a** respectively). It is important to mention, that after the MMT deprotection, the freed amine is protonated and requires quenching with a base to react it further or store immobilized peptide for longer periods of time. Like **4a** and **5a**, the immobilized progenitors were subjected to the same sequence of events leading to the isolation of the C-terminal monofunctionalized TLR2 conjugates (**6a, b**) and TLR7 conjugates (**7a**) in satisfactory yields.

Now that the stage is reached where all mono-conjugates are synthesized and the needed experimental protocols for ligand incorporation are available, the assemblage of the four target dual conjugates can be undertaken using immobilized protected peptides (**12a, b**). Both the TLR2 and the TLR7 were appended at the N-terminal end of **12a** and **12b** to give the monofunctionalized resins (**14a, b**) and (**15a, b**) respectively. The TLR2 ligand was installed at the C-terminal end of resin (**14a, b**) and the TLR7 ligand was installed at the C-terminal end of resin (**15a, b**) using the same protocols as described above. Finally, the same isolation used for the monoconjugates was successful for the isolation of all dual conjugates to give $DEVA_5K$ dual conjugates **1a** and **2a** in a yield of 5% and 2% respectively, and HAAHA dual conjugates **1b** and **2b** in a yield of 2% and 4% respectively.

Immunological evaluation

Until now, only conjugates **1a** and **1b** were tested for their ability to induce DC maturation by determining the expression of surface markers CD40 CD70, CD80 and CD86 and IL-12 cytokine production in bone marrow-derived dendritic cells (BMDCs). Conjugates **2a** and **2b** were left out this evaluation, since their immunological relevance was questionable after *Gential et al.* published that C-terminal TLR7 functionalized conjugates failed to induce any significant T-cell responses.[35] Dual conjugates were compared with its reference compounds to investigate any synergistic interactions between TLR2 and TLR7. In addition, mixtures containing TLR7 ligand **8**, TLR2 ligand **11**, both **8** and **11**, and reference conjugates **5a, b** and **6a, b** with TLR2 ligand **11** and TLR7 **8** respectively were incubated with BMDCs. Finally, peptides **3a** and **3b** served as a

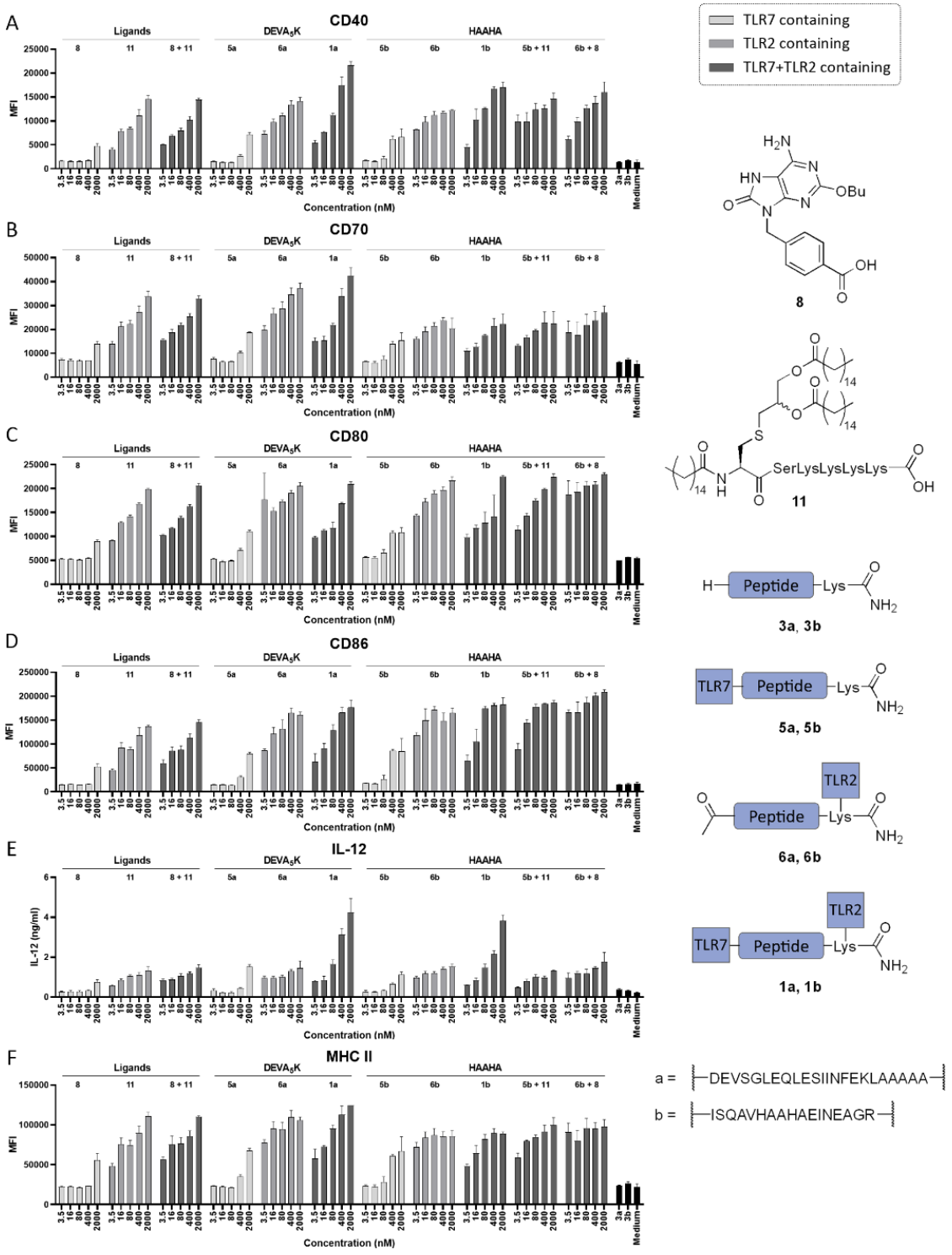


Figure 2: Induction of BMDC maturation by TLR2-TLR7 functionalized conjugates **1a** and **1b** with reference compounds **3a**, **3b**, **5a**, **5b**, **6a**, **6b**, **8** and **11** as measured by the expression of CD40 (A), CD70 (B), CD80 (C), CD86 (D), MHC II (F) and the production of IL-12 (E). BMDCs were incubated with either ligand, conjugate, or ligand plus conjugate of interested. After 3 h, the cells were washed and incubated with fresh medium. After another 16 h, the supernatant was harvested and analysed for IL-12 production by ELISA analysis. Additionally, the cells were harvested and stained with an appropriate fluorescent antibody for CD40, CD70, CD80, CD86 and MHC II. Expression levels could then be determined by flow cytometry. Sequences for MHC I epitope containing peptide DEVA$_5$K (**a**) and MHC II epitope containing peptide HAAHA (**b**) are given in the bottom right of the figure.

negative control. By comparing the data of these control experiments with those of the dual conjugates, the effect of the conjugation of both ligands to the antigenic peptides and potential synergistic effects of the ligands could be determined.

Overall, biological evaluation of dual conjugates **1a** and **1b** show upregulation of surface markers comparable to all other conditions where the TLR2 ligand is present, either as free ligand or as a monoconjugate (see Figure 2A-D and F). This is consistent with the fact that significantly less upregulation is observed when the dendritic cells are exposed to the TLR7 or the TLR7 monoconjugate. Monoconjugates of ligand **8** and **11** with both antigens display slightly higher activities as does co-stimulation of both TLR2 and TLR7. The same trend is observed for IL-12 production, however treatment with **1a** and **1b** elicited by far the strongest IL-12 productions out of all incubation conditions (see Figure 2E). Interestingly, as the conjugation of either TLR2 or TLR7 ligands to the antigen or their synergistic interaction show only small increases in cytokine production, the nature for its increase should find its origin elsewhere. It could be that activation of both TLR2 and TLR7 in proximity induces stronger synergistic activation where in this case the antigen facilitates the clustering of receptors as it serves as a bridge between both agonists. Though TLR2 primarily resides on the cell membrane and TLR7 inside endosomes, it has been reported that TLR2 is internalized through endosomes[40-41] and thus being in proximity. Furthermore, *Mancini et al.*[8] reported a similar increase in activity when covalent coupling a TLR2 and TLR9 ligand together with an PEG based linker.

In conclusion, the newly designed dual conjugates comprising either model peptide $DEVA_5K$ (**1a, b**) or HAAHA (**2a, b**) and functionalized at the C- and N-terminal ends with a TLR2 and TLR7 ligand were successfully assembled via a combination of automatic and manual SPPS procedures, utilizing Fmoc-chemistry elongation protocol. Functionalization of the C-terminal end was attained using a lysine building block having its side chain orthogonally protected with the very acid sensitive MMT group. Biological evaluation of the dual conjugates **1a** and **1b** showed an upregulation of surface markers CD40, CD70, CD80, CD87 comparable to mono Pam_3 functionalized conjugates but elicited a superior IL-12 production. This increase could be explained by the clustering of activated TLR2 and TLR7 as the mixtures of the mono-conjugates and simultaneous activation of the receptors do not produce similar results. To determine whether the receptor clustering is the driving force in the increased agonistic activity, a similar experiment could be performed using a construct where both ligands are connected with a polyethylene glycol spacer, similarly to the approach of *Esser-Kahn et al.*[8] In the case that purely the conjugation drives increased activity, one could also investigate what the optimal length of the spacer would be and use this information to optimize self-adjuvating vaccine designs.

Experimental

Synthesis of simplified TLR2 ligand conjugated neoepitope containing synthetic long peptides

General Information

All reagents and solvents used in the solid phase peptide synthesis were purchased from Biosolve (Netherlands). Fmoc amino acids building blocks were purchased from Sigma Aldrich or Novabiochem. Tentagel based resins were purchased from Rapp Polymere GmbH (Germany). The solid-phase peptide synthesis was performed on a TRIBUTE™ Peptide Synthesiser (Gyros Protein Technologies AB, Arizona, USA) applying Fmoc based protocol starting with Tentagel S RAM resin (0.22-0.25 mmol/g) on a 100 µmol scale using established Fmoc protocols. LC-MS analysis was performed on one of the following LC-MS systems: A Thermo Finnigan LCQ Advantage MAX ion-trap mass spectrometer with an electrospray ion source coupled to Surveyor HPLC system (Thermo Finnegan), A Thermo Finnigan LCQ Fleet MAX ion-trap mass spectrometer with an electrospray ion source coupled to Vanquish UPLC system (Thermo Finnegan) or an Agilent Technologies 1260 Infinity LC system (detection simultaneously at 214 and 254 nm) coupled to a Agilent Technologies 6120 Quadrupole MS. Using an analytical Phenomenex Gemini® (3 µm C_{18} 110 Å 50x4.6 mm), Vydac 219TP 5 µm (150x4.6mm (Phenomex, 50 x 4.60 mm, 3 microns) or Cosmosil 5C_4-MS (5 µm particle size, 150x4.6 mm) in combination with eluents A: H_2O; B: MeCN and C: 1% TFA (aq.) as the solvent system, in which the gradient was modified by changing the ratio of A in B in combination with 10% C. High resolution mass spectra were recorded on an Q-Exactive HF Orbitrap (Thermo Scientific) equipped with an electrospray ion source (ESI), injection of 2 µL of a 1 µM solution via Ultimate 3000 nano UPLC (Dionex) system, with an external calibration (Thermo Scientific); Source voltage of 3.5 kV, capillary temperature 275 °C, no sheath gas, resolution = 240.000 at m/z = 400. Mass range m/z = 160-2000 or to a maximum of 6000. Eluents used: MeCN:H_2O (1:1 v/v) supplemented with 0.1% formic acid.

General procedure for automated solid phase synthesis

All reagents and solvents used in the solid phase peptide synthesis were purchased from Biosolve (Netherlands). Fmoc amino acids building blocks were purchased from Sigma Aldrich or Novabiochem. Tentagel based resins were purchased from Rapp Polymere GmbH (Germany). The solid-phase peptide synthesis was performed on a TRIBUTE® Peptide Synthesiser (Gyros Protein Technologies AB, Arizona, USA) applying Fmoc based protocol starting with Tentagel S-RAM resin (0.22-0.25 mmol/g) on a 100 µmol scale using established Fmoc protocols. The consecutive steps preformed during each cycle were:

1. Deprotection of the Fmoc protecting group with 3 x 4 mL 20% piperidine in DMF for 3 min;
2. Wash, 3 x 4 mL DMF;
3. First coupling of the appropriate amino acid applying a five-fold excess. Generally, the Fmoc amino acid (0.5 mmol, 5 eq.) building block was dissolved 0.2 M HCTU in DMF (2.5 mL, 5 eq.) in its loading cartridge and the resulting solution was transferred to the reaction vessel. Next, the cartridge was washed with a 0.5 M D*i*PEA in DMF (2 mL, 10 eq.) and subsequently the solution was transferred to the reaction vessel. The reaction vessel was shaken for 1 h at RT;
4. Wash, 3 x 4 mL DMF;
5. Second coupling of the appropriate amino acid applying a five-fold excess. Generally, the Fmoc amino acid (0.5 mmol, 5 eq.) building block was dissolved 0.2 M HCTU in DMF (2.5 mL, 5 eq.) in its loading cartridge and the resulting solution was transferred to the reaction vessel. Next, the

cartridge was washed with a 0.5 M D*i*PEA in DMF (2 mL, 10 eq.) and subsequently the solution was transferred to the reaction vessel. The reaction vessel was shaken for 1 h at RT;

6. Wash, 3 x 4 mL DMF;
7. Capping of unreacted peptide with 1 x 10% Ac_2O in DMF solution (5 mL) for 3 min;
8. Wash, 3 x 4 mL DMF;

After the last coupling cycle, the final Fmoc group was deprotected with 3 x 4 mL 20% piperidine in DMF for 3 min. Finally, the resin was washed with DMF (3x) and DCM (3x) and dried using a N_2 flow.

The following amino acid building blocks were used for the synthesis: Fmoc-Ala-OH, Fmoc-Asn(Trt)-OH, Fmoc-Asp(*t*Bu)-OH, Fmoc-Arg(Pbf)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Gln(Trt)-OH, Fmoc-Glu(*t*Bu)-OH, Fmoc-Gly-OH, Fmoc-His(Trt)-OH, Fmoc-Ile-OH, Fmoc-Leu-OH, Fmoc-Lys(Boc)-OH, Fmoc-Met-OH, Fmoc-Phe-OH, Fmoc-Pro-OH, Fmoc-Ser(*t*Bu)-OH, Fmoc-Thr(*t*Bu), Fmoc-Trp(Boc)-OH Fmoc-Tyr(*t*Bu)-OH, Fmoc-Val-OH, Fmoc-Abu-OH.

General procedure capping of N-terminal end peptide 12a and 12b

25 µmol of immobilized peptide was taken and treated with a solution of 10% Ac_2O and 5% NMM in DMF for shaken for 5 min in a reaction syringe. Afterwards, the reaction syringe was drained, and the resin was washed with DMF (3x) and DCM (6x) and dried under a N_2 flow.

General procedure palmitoyl-Cys((*RS*)-2,3-di(palmitoyloxy)-propyl)-OH installation

25 µmol of SK_4 elongated peptide or conjugate was taken and treated with a solution of Palmitoyl-N-(2,3-bis(palmitoyloxy)propyl)-L-cysteine (50 µmol, 44.7 mg, 2 eq.) and HCTU (50 µmol, 20.9 mg, 2 eq.) in 1:1 DCM:DMF (0.75 mL). D*i*PEA (50 µmol, 8.7 µL, 2 eq.) was added and the reaction syringe was shaken for 15 min. A second portion of D*i*PEA (50 µmol, 8.7 µL, 2 eq.) was added and the reaction syringe was shaken overnight. The next morning the resin was washed with DMF (3x) and DCM (6x) and dried under a N_2 flow.

General procedure MMT removal

25 µmol of MMT protect peptide or conjugate was taken and treated multiple times, first 10x 30 seconds and second 2x 5 minutes, with a 1% TFA in DCM solution (4 mL). After each treatment, the resin was washed with DCM and finally wash 6x with DCM. Important! Before coupling the next moiety, the freed side chain, the resin must be washed with 20% D*i*PEA in NMP.

General procedure (2-(2-(Fmoc-amino)ethoxy)ethoxy)acetic acid installation

25 µmol of peptide or deprotected amine was treated with a solution of (2-(2-(Fmoc-amino)ethoxy)ethoxy)acetic acid (50 µmol, 19.3 mg, 2 eq.), HCTU (50 µmol, 20.9 mg, 2 eq.), D*i*PEA (100 µmol, 34.8µL, 4 eq.) in DMF (0.75 mL) and shaken overnight. The next morning the resin was washed with DMF (3x) and DCM(6x) and dried under a N_2 flow.

General procedure 1-(4-((6-amino-2-butoxy-8-oxo-7,8-dihydro-9H-purin-9-yl)methyl)phenyl)-1-oxo-5,8-dioxa-2-azadecan-10-acid installation

25 µmol of peptide or deprotected amine was treated with a solution of 1-(4-((6-amino-2-butoxy-8-oxo-7,8-dihydro-9H-purin-9-yl)methyl)phenyl)-1-oxo-5,8-dioxa-2-azadecan-10-acid (50 µmol, 22.9 mg, 2 eq.), HCTU (50 µmol, 20.9 mg, 2 eq.), D*i*PEA (100 µmol, 34.8µL, 4 eq.) in DMF (0.75 mL) and

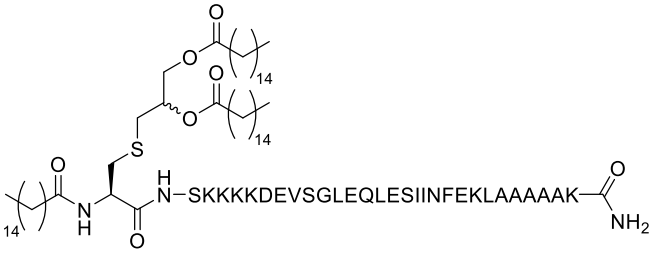
shaken overnight. The next morning the resin was washed with DMF (3x) and DCM (6x) and dried under a N_2 flow.

General procedure final deprotection and purification

25 µmol of peptide or conjugate containing resin was deprotected using a solution of 95:2.5:2.5 TFA:H_2O:TIS (3 mL) for 105 min. The deprotection solution was added to a centrifuge tube containing 46 mL of 1:1 Et_2O:Pentane and cooled at -20 ˚C. The tube was centrifuged for 15 min, the organic solution was decanted, and the remaining pallet was dried using a N_2 flow. Sequences containing a histidine moiety were redissolved in a solution of 1:1:1 HO*t*Bu:H_2O:MeCN and lyophilized overnight. The crude products were purified using HPLC.

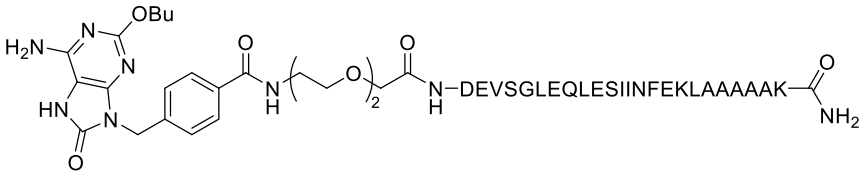
H-Asp-Glu-Val-Ser-Gly-Lue-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Ala-Ala-Ala-Ala-Ala-Lys-NH_2 (3a)

6% yield (3.96 mg, 1.56 µmol). **LC-MS:** R_t = 8.533 (Vydac 219TP 5 µm (150x4.6mm) Diphenyl, 10-90% MeCN, 21 min); **ESI-MS:** m/z 1273.8 $[M+2H^+]^{2+}$; **HRMS [M+3H]$^{3+}$:** $[C_{112}H_{185}N_{29}O_{38}]^{3+}$ 849.12193 (measured), 849.12179 (calculated).



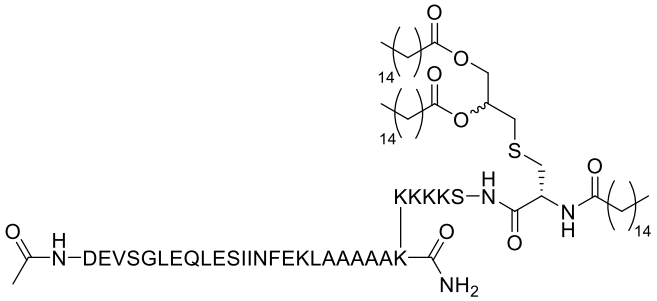
Palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-Ser-Lys-Lys-Lys-Lys-Asp-Glu-Val-Ser-Gly-Lue-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Ala-Ala-Ala-Ala-Ala-Lys-NH_2 (4a)

4% yield (5.91 mg, 2.22 µmol). **LC-MS:** R_t = 12.221 (Vydac 219TP 5 µm (150x4.6mm) Diphenyl, 10-90% MeCN, 21 min); **ESI-MS:** m/z 1347.2 $[M+3H^+]^{3+}$;



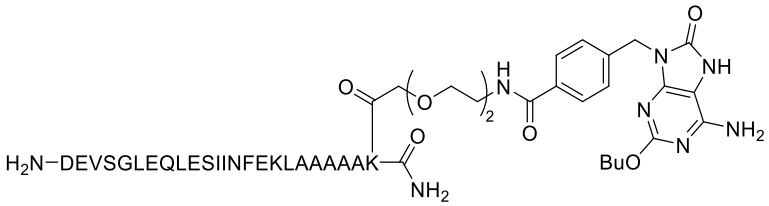
1-(4-((6-amino-2-butoxy-8-oxo-7,8-dihydro-9H-purin-9-yl)methyl)phenyl)-1-oxo-5,8-dioxa-2-azadecan-10-amide-Asp-Glu-Val-Ser-Gly-Lue-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Ala-Ala-Ala-Ala-Ala-Lys-NH_2 (5a)

2% yield (1.28 mg, 0.49 µmol). **LC-MS:** R_t = 5.25 (Phenomenex Gemini® 3 µm C_{18} 110 Å 50x4.6 mm, 10-90% MeCN, 10 min); **ESI-MS:** m/z 1515.8 $[M+2H^+]^{2+}$; **HRMS [M+3H]$^{3+}$:** $[C_{135}H_{216}N_{35}O_{44}]^{3+}$ 1010.52322 (measured), 1010.52413 (calculated).



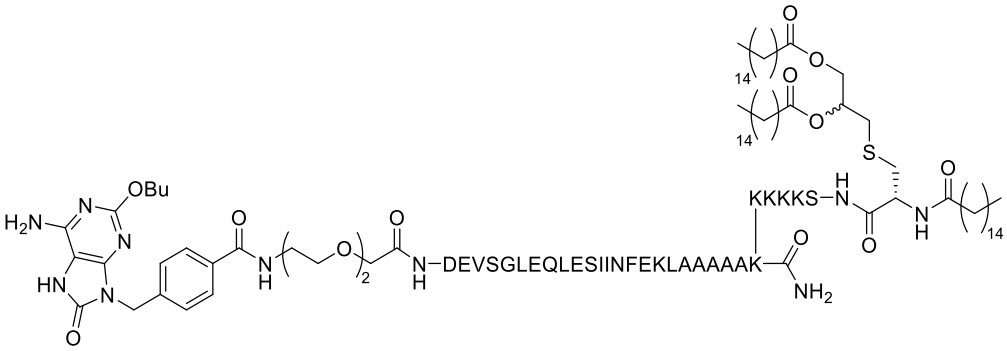
Acetyl-Asp-Glu-Val-Ser-Gly-Lue-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Ala-Ala-Ala-Ala-Ala-Lys(Palmitoyl- Cys((RS)-2,3-di(palmitoyloxy)-propyl)-Ser-Lys-Lys-Lys-Lys)-NH_2 (6a)

4% yield (4.53 mg, 1.11 µmol). **LC-MS:** R_t = 12.221 (Vydac 219TP 5 µm (150x4.6mm) Diphenyl, 10-90% MeCN, 21 min); **ESI-MS:** m/z 1360 $[M+3H^+]^{3+}$; **HRMS [M+4H]$^{4+}$:** $[C_{195}H_{341}N_{39}O_{51}S]^{4+}$ 1020.38177 (measured), 1020.38251 (calculated).



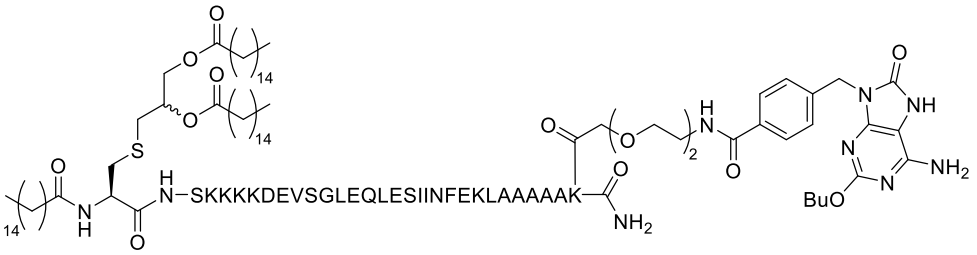
Acetyl-Asp-Glu-Val-Ser-Gly-Lue-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Ala-Ala-Ala-Ala-Ala-Lys(1-(4-((6-amino-2-butoxy-8-oxo-7,8-dihydro-9H-purin-9-yl)methyl)phenyl)-1-oxo-5,8-dioxa-2-azadecan-10-amide)-NH_2 (7a)

4% yield (2.97 mg, 0.97 µmol). **LC-MS:** R_t = 5.43 (Phenomenex Gemini® 3 µm C_{18} 110 Å 50x4.6 mm, 10-90% MeCN, 10 min); **ESI-MS:** m/z 1536.9 $[M+2H^+]^{2+}$; **HRMS [M+3H]$^{3+}$:** $[C_{137}H_{218}N_{35}O_{45}]^{3+}$ 1024.52767 (measured), 1024.52765 (calculated).



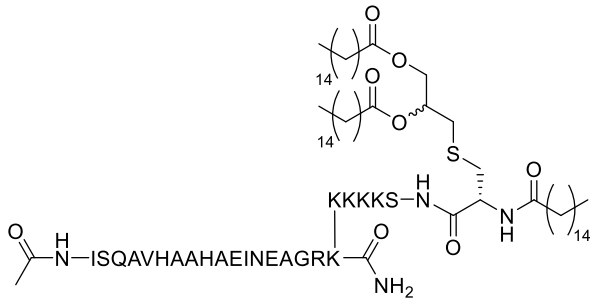
1-(4-((6-amino-2-butoxy-8-oxo-7,8-dihydro-9H-purin-9-yl)methyl)phenyl)-1-oxo-5,8-dioxa-2-azadecan-10-amide-Asp-Glu-Val-Ser-Gly-Lue-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Ala-Ala-Ala-Ala-Ala-Lys(Palmitoyl- Cys((RS)-2,3-di(palmitoyloxy)-propyl)-Ser-Lys-Lys-Lys-Lys)-NH_2 (1a)

5% yield (5.18 mg, 1.15 µmol). **LC-MS:** R_t = 12.48 (Vydac 219TP 5 µm (150x4.6mm) Diphenyl, 10-90% MeCN, 21 min); **ESI-MS:** m/z 1508.2 $[M+3H^+]^{3+}$; **HRMS [M+5H]$^{5+}$:** $[C_{216}H_{372}N_{45}O_{56}S]^{7+}$ 904.94672 (measured), 904.94676 (calculated).



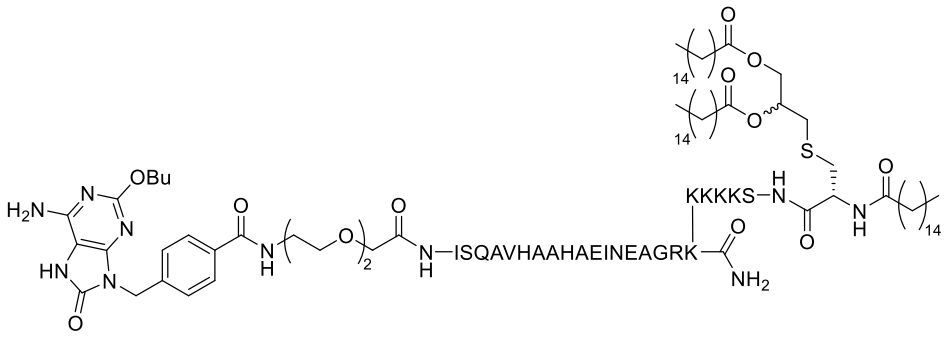
Palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-Ser-Lys-Lys-Lys-Lys-Asp-Glu-Val-Ser-Gly-Lue-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Ala-Ala-Ala-Ala-Ala-Lys(1-(4-((6-amino-2-butoxy-8-oxo-7,8-dihydro-9H-purin-9-yl)methyl)phenyl)-1-oxo-5,8-dioxa-2-azadecan-10-amide)-NH_2 (2a)

2% yield (2.81 mg, 0.62 µmol). **LC-MS:** R_t = 13.09 (Vydac 219TP 5 µm (150x4.6mm) Diphenyl, 10-90% MeCN, 21 min); **ESI-MS:** m/z 1508.3 $[M+3H^+]^{3+}$; **HRMS [M+6H]$^{6+}$:** $[C_{216}H_{373}N_{45}O_{56}S]^{6+}$ 754.29018 (measured), 754.29011 (calculated).



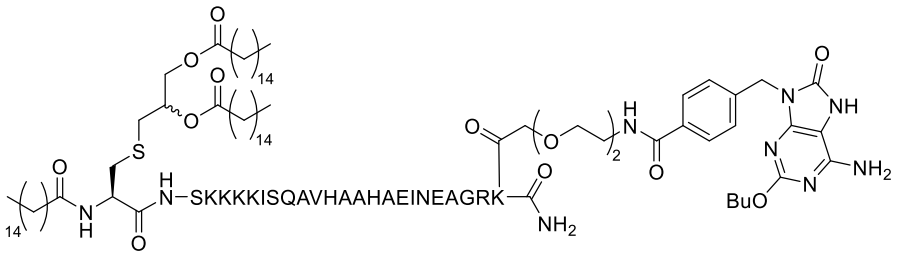
Acetyl-Ile-Ser-Gln-Ala-Val-His-Ala-Ala-Hys-Ala-Glu-Ile-Asn-Glu-Ala-Gly-Arg-Lys(Palmitoyl- Cys((RS)-2,3-di(palmitoyloxy)-propyl)-Ser-Lys-Lys-Lys-Lys)-NH_2 (6b)

3% yield (2.54 mg, 0.74 µmol). **LC-MS:** R_t = 8.54 (Phenomenex Gemini® 3 µm C_4 110 Å 50x4.6 mm, 10-90% MeCN, 10 min); **ESI-MS:** m/z 1718.3 $[M+2H^+]^{2+}$; **HRMS [M+5H]$^{5+}$:** $[C_{163}H_{294}N_{39}O_{38}S]^{5+}$ 687.63900 (measured), 687.63931 (calculated).



1-(4-((6-amino-2-butoxy-8-oxo-7,8-dihydro-9H-purin-9-yl)methyl)phenyl)-1-oxo-5,8-dioxa-2-azadecan-10-amide-Ile-Ser-Gln-Ala-Val-His-Ala-Ala-Hys-Ala-Glu-Ile-Asn-Glu-Ala-Gly-Arg-Lys(Palmitoyl- Cys((RS)-2,3-di(palmitoyloxy)-propyl)-Ser-Lys-Lys-Lys-Lys)-NH_2 (1b)

Performed on a 50 µmol. 2% yield (3.31 mg, 0.85 µmol). **LC-MS:** R_t = 8.54 (Phenomenex Gemini® 3 µm C_4 110 Å 50x4.6 mm, 10-90% MeCN, 10 min); **ESI-MS:** m/z 1939.2 $[M+2H^+]^{2+}$; **HRMS [M+6H]$^{6+}$:** $[C_{184}H_{321}N_{45}O_{43}S]^{6+}$ 646.90049 (measured), 646.90005 (calculated).



Palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-Ser-Lys-Lys-Lys-Lys-Ile-Ser-Gln-Ala-Val-His-Ala-Ala-Hys-Ala-Glu-Ile-Asn-Glu-Ala-Gly-Arg-Lys(1-(4-((6-amino-2-butoxy-8-oxo-7,8-dihydro-9H-purin-9-yl)methyl)phenyl)-1-oxo-5,8-dioxa-2-azadecan-10-amide)-NH_2 (2b)

Performed on a 50 µmol. 4% yield (7.77 mg, 2.00 µmol). **LC-MS:** R_t = 8.39 (Phenomenex Gemini® 3 µm C_4 110 Å 50x4.6 mm, 10-90% MeCN, 10 min); **ESI-MS:** m/z 1939.8 $[M+2H^+]^{2+}$; **HRMS $[M+5H]^{5+}$:** $[C_{184}H_{320}N_{45}O_{43}S]^{5+}$ 717.18531 (measured), 717.18533 (calculated).

In vitro experiment[42] (work of E. Tondini)

Cell culture.

Cell culture. Bone marrow-derived dendritic cells were differentiated from bone marrow stem cells that were harvested from the femurus and tibia of C57BL/6 mice and cultured in IMDM (Lonza) supplemented with FCS (Greiner) , Glutamax (Gibco), penicillin (Gibco), β-mercaptoethanol (Merck) and R1 supernatant. R1 supernatant was obtained by culturing NIH3T3 fibroblasts transfected with GM-CSF. The B3Z and OTIIZ cell lines were cultured in IMDM medium (Lonza supplemented with 8% FCS (Greiner), penicillin and streptomycin, glutamine (Gibco) β-mercaptoethanol (Merck), and hygromycin B (AG Scientific Inc, San Diego, CA, USA) to maintain expression of the beta-galactosidase reporter gene

In vitro DC maturation assay

The test compounds were dissolved in DMSO at a concentration of 1 mM and sonicated in water bath for 15 min. Murine bone marrow-derived dendritic cells were seeded in 96-well plates at a density of 50.000 cells/well and incubated with titrated amounts of compounds. After 3 h of incubation, the cells were washed once and incubated with fresh medium. After 16 h, supernatant was harvested for ELISA analysis (Biolegend) to measure the amount of produced IL-12p40. The cells were harvested and stained with fluorescently labeled antibodies directed against CD40, CD70, CD80, CD86 and MHCII, after which expression levels were determined by flow cytometry.

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