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

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Chapter 6

Urea-derived Pam_3CSK_4 derivatives as new agonists for TLR1/2

6.1 Introduction

Lipoproteins that are part of the outer membrane of gram-positive and gram-negative bacteria share the unusual *N*-terminal amino acid S-(2,3-dihydroxypropyl)-L-cysteine, acylated by fatty acids. The pattern recognition receptor of the innate immune system for these lipoproteins is TLR2 and it was discovered that activation of TLR2 can be attained with S-(2,3-bispalmitoxyloxypropyl)-Cys-Ser-Ser-Asp-Ala (Pam_2CSSNA, **1**), a structure that originally was isolated from natural sources.[1,2] On the basis of this finding, a series of lipopeptide derivatives have been synthesized to assess structure-activity relations (SAR) for optimal ligand recognition.[1,3] These studies led to the synthesis of a number of derivatives of **1** bearing two to five amino acids that showed an activity comparable to the natural lipoprotein.[3-8] In contrast, truncated derivatives of **1** bearing only one or no amino acid were found to be inactive. These results suggest that elongation of the S-(2,3-

bispalmitoxyloxypropyl)-*N*-palmitoyl-Cys moiety by an oligopeptide of a certain length and composition is necessary for immunological activity.
In this framework Reitermann reported the synthesis and immunological evaluation of a series of triacylated lipopeptide derived ligands, including Pam_3CSSNA, Pam_3CSK_4, Pam_3CAG and Pam_3CS.[9] It appeared that Pam_3CSK_4 (*Figure 1*, **2**) was the most potent immune adjuvant out of this series with the additional advantage of improved solubility in water. These advantageous properties have made Pam_3CSK_4 one of the most applied TLR2 ligands.[10,11]

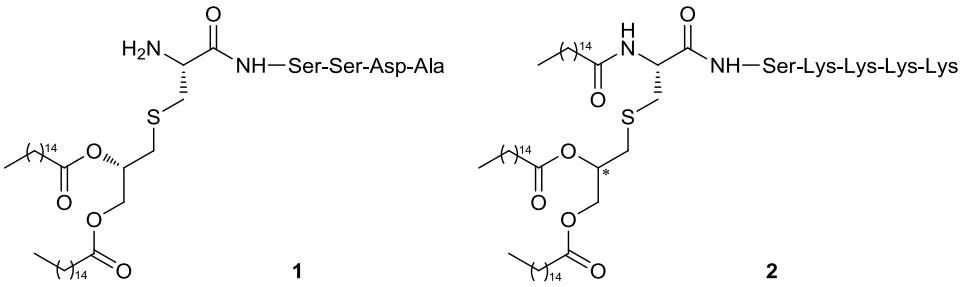


Figure 1. TLR2-L Pam_2CSSNA (**1**) and TLR2-L Pam_3CSK_4 (**2**).

Next to these studies, the structures of TLRs in the absence and in complex with the corresponding ligands have been investigated with X-ray crystallography. It turned out that TLR2 is a unique member of the TLR family as it can form heterodimers with TLR1 and TLR6.[12-14] The heterodimer combination determines the ligand specificity and the TLR2/6 complex recognizes the di-acylated ligand Pam_2CSK_4, whereas the TLR1/2 complex recognizes tri-acylated lipopeptides such as Pam_3CSK_4 (**2**). The crystal structure of the TLR1/2 heterodimer co-crystallized with the Pam_3CSK_4 ligand (**2**) (*Figure 2A*), as reported by Jin *et al*.[12] showing that the two fatty esters at the glycerol moiety in **2** interact with a pocket formed at the convex side of the TLR2 protein while the *N*-terminal fatty chain fits in the hydrophobic site of the TLR1 protein.
Overall, binding of Pam_3CSK_4 with the receptor results in an M-shaped TLR1/2 dimer that is further stabilized by internal hydrogen-bonding and ionic interactions. Inspection of the crystal structure of the TLR1/2 heterodimer co-crystallized with the Pam_3CSK_4 ligand reveals that an extra hydrogen bridge with the oxygen of the phenyl alanine-312 residue can be formed (*Figure 2A, yellow dotted line*) by substitution of the α-CH_2 of a fatty acid amide in a ligand with a NH, resulting in the urea derivative UPam-X1 in which X1 is the natural occurring L-serine (*Figure 2B*). It was hypothesized that the potential formation of an additional hydrogen bridge by UPam-X1 would lead to an improved TLR2 agonist.

It is also not excluded that replacement of serine by another amino acid would further improve the binding of the ligand with the receptor.

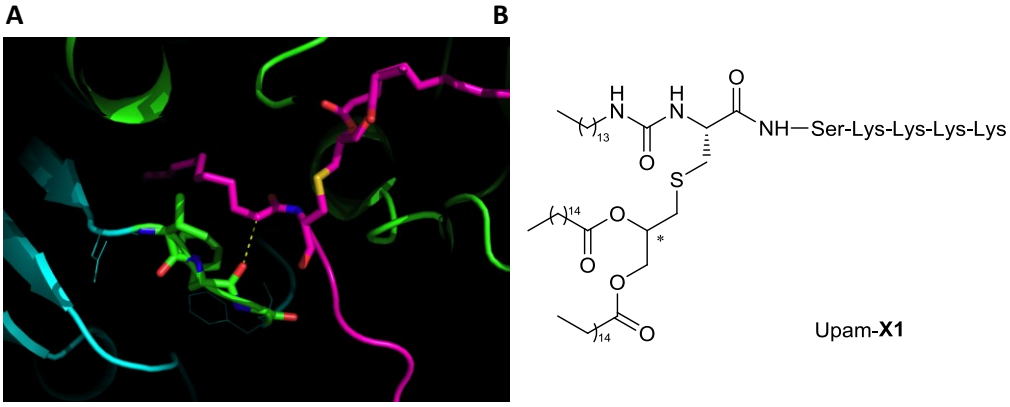


Figure 2. A) TLR1/2 co-crystallized with Pam_3CSK_4; B) **$UPam_3SK_4$** (**Upam-X1**).

To verify these assumptions a small library of UPam-**Xn** derivatives (**Xn** = **X1** – **X18**) was projected in which the serine in modified Upam-**X1** is replaced by a number of natural and non-proteinogenic amino acids **X2** – **X18** (*Figure 3*). Alongside serine (**X1**) the other amino acids were selected on the basis of differences in the nature, length or bulkiness of their side chains. The frequently used non-natural isostere of cysteine, 2-aminobutanoic acid (**X2**) may function as serine replacement.[15] The length of the aliphatic side chain was increased further by the use of norvaline (**X3**) and norleucine (**X4**). Allylglycines (**X5** and **X6**) and propargylglycine (**X7**) were chosen by virtue of their unsaturated carbon chains. To evaluate the effect of an amine and amide in the side chain 2,4-diaminobutanoic acid **X8** and asparagine **X9** were selected. The library contains several thio amino acids: L- and D-cysteine (**X10** and **X11**) as well as the more bulky thio amino acids 3-mercaptovaline (**X12**), 3-thienylalanine (**X13**) and 2-thienylglycine (**X14**). To investigate the effect of a missing chain, glycine (**X15**) was implemented. To study the effect of an extended peptide part beta-alanine **X16** and beta-lysine **X17** were employed. Finally, naphtyl alanine (**X18**) was selected as a negative control, because the bulky aromatic side chain was expected to not fit in the active side.

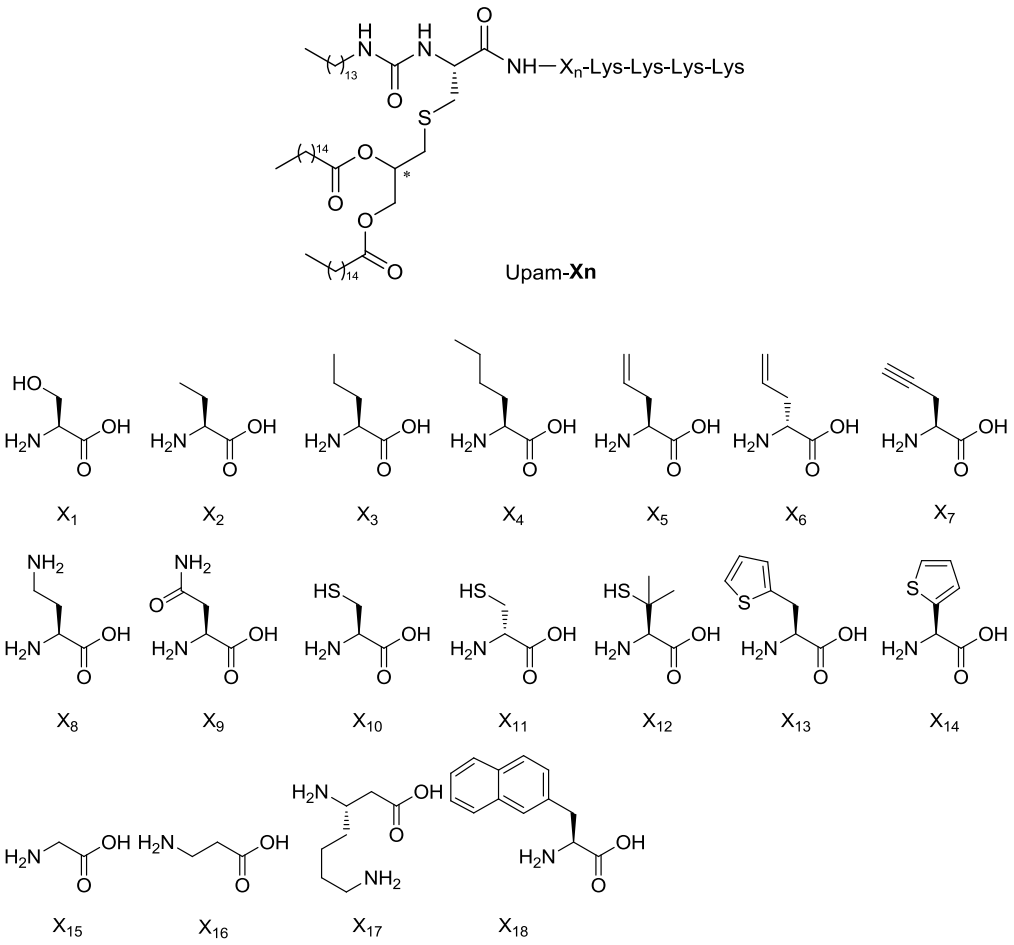


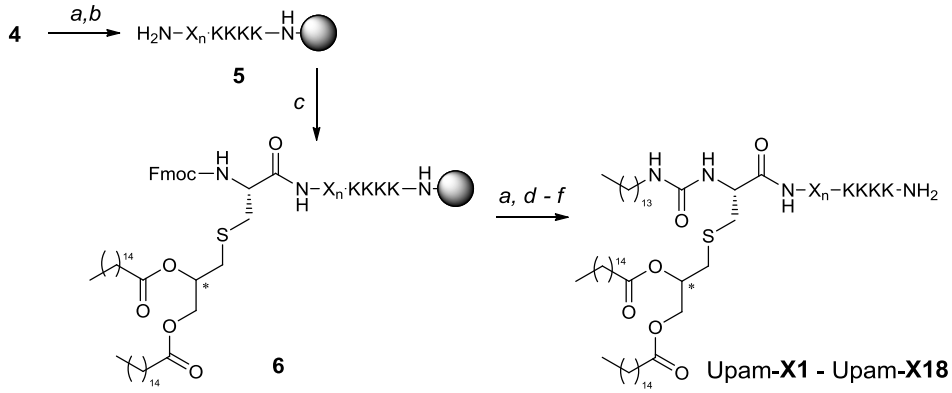
Figure 3. The Upam-**Xn** library with the amino acids **X1** – **X18**.

Since it is known that conjugates comprising a TLR2 ligand covalently linked to a peptide epitope exhibit an improved immunological profile[16] in comparison with a mixture of the individual components, the synthesis and evaluation of conjugate **7**, comprising UPam-**X1** covalently linked to the epitope $DEVA_5K$, is also described.

6.2.1 Synthesis of $UPamSK_4$ and library

The library of eighteen members (Upam-**X1** – Upam-**X18**) was constructed with the aid of a SPPS protocol (*Scheme 1*) using the following commercially available items: solid support S Ram Tentagel **4**, Fmoc protected amino acids equipped with standard acid labile protective groups, coupling agent HCTU, Fmoc-Cys((RS)-2,3-di(palimitoyloxy)-propyl)-OH and tetradecyl isocyanate.
In the first instance, attention was directed to the synthesis of the Pam_3Cys urea derivative with the natural occurring L-serine resulting in ligand Upam-**X1** (**X1** = L-serine). The fully automated peptide synthesis was started with the elongation of Fmoc protected S Ram Tentagel **4** to the SK_4-pentapeptide **5** using a standard three step coupling cycle, consisting of HCTU mediated peptide bond formation, capping with acetic anhydride and removal of the Fmoc-group by treatment with piperidine. Next, the Fmoc-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-OH moiety was condensed with resin **5** under influence of PyBOP and DiPEA by a manual procedure resulting in immobilized ligand **6**. The synthesis was followed by the removal of the *N*-terminal Fmoc group and reaction of the released amine with an excess of tetradecyl isocyanate for 18 h to give the immobilized fully protected precursor of UPam-**X1**. Removal of the protective groups and cleavage from the solid support was accomplished by treatment with a cocktail of 95% TFA, 2.5% TIS and 2.5% H_2O for 104 minutes and subsequent precipitation with Et_2O and finally the purification by RP-HPLC to result in the isolation ligand Upam-**X1** in 6% overall yield.
Guided by this result the remaining seventeen members of the projected library (Upam-**X2** – Upam-**X18**) were prepared in a parallel synthesis approach. To achieve this, the immobilized tetrapeptide K_4 was synthesized on 1 mmol scale, using the above described automated SPPS protocol. The resin with immobilized K_4 was divided in portions of 20 umol and each portion was manually condensed with one of the intended amino acids **X2** – **X18**. All the seventeen immobilized pentapeptides were subsequently manually elongated according to the above described procedure to give immobilized and protected precursors of the target UPam-**X2** – Upam-**X18** derivatives. Treatment with the deprotection and cleavage conditions as described above and RP-HPLC purification resulted in the isolation of seventeen UPam derivatives (**X2** – **X18**) in 6% – 27% overall yield.

Scheme 1. Synthesis UPam-**X1 –** Upam-**X18**.



Reaction conditions: *a*) 20% piperidine, NMP; *b*) Fmoc SPPS cycle for K_4 followed by appropriate amino acid **X1 – X18**; *c*) Fmoc-Cys((RS)-2,3-di(palmitoyloxy)-propyl)-OH, PyBOP, DiPEA; *d*) tetradecyl isocyanate, NMP; *e*) 95% TFA, 2.5% TIS, 2.5% H_2O; *f*) RP-HPLC.

6.2.2 Immunological evaluation of TLR1/2 ligand and library

To examine the potency of the obtained eighteen urea-derived Upam-**X1** – Upam-**X18** derivatives the compounds were submitted to a series of immunological assays similar to those described in the previous chapters. The potency of the urea-derived library was probed against the parent TLR2-L Pam_3CSK_4 (**2**). The maturation properties of the compounds were evaluated by measuring the IL-12p40 production of D1 DCs that were exposed to the UPam derivatives. To get additional information on the maturation process of DCs the upregulation of cell surface markers CD40, CD80 and MHC Class II was assessed.

The results of the DC maturation assay are depicted in *Figure 4*. It is noticed that all assay data result in a bell-shaped curve which is probably due to a saturation effect of the tested ligands. The newly synthesized ligand UPam-**X1** turned out to mature the DC better than the unmodified Pam_3CSK_4 **2** and therefore UPam-**X1** was subsequently used as a reference compound to evaluate the maturing capacity of the other UPam derivatives. The results of the modification with amino acids **X2**, **X5**, **X7** – **X9** containing small, mostly hydrophobic side chains look promising. From all derivatives 2-aminobutanoic acid (**X2**), containing an ethyl group instead of a hydroxymethyl proved to be most active. Longer alkyl chains such as propyl (**X3**) and butyl (**X4**) were less beneficial, while bulky side-chains were detrimental for the immunostimulatory activity (**X12**, **X18**). The UPam derivatives with unsaturated side chains such as in **X5** and **X7** were more active than the saturated counterpart **X3**, but did not quite reach the activity of **X2**. Interestingly, the results of ligands with amino acids **X8** and **X9** bearing side chains capable of forming hydrogen bridges were contradictive: **X9** was found fairly active whereas **X8** showed an activity close to **X2**. When allylglycine containing UPam ligand (**X5**) was compared with the corresponding D-amino acid containing derivative **X6** it was shown that D-allyl glycine derived ligand **X6** showed barely any activity. This is in line with previously reported findings that amino acids with a D-configuration are inactive.[17] A similar drop in activity was observed for D- and L-cysteine, **X10** and **X11**, respectively. Amino acids with thiol containing side chains **X10** – **X14** showed no activity below a concentration of 300 pM, while the most promising compounds **X2** and **X8** were still active at 30 pM concentration. The substitution with glycine (**X15**) gave a lower activity than **X1** and compounds with beta-alanine (**X16**) and beta-lysine (**X17**) modifications were almost inactive. The side chain in the beta-substituted amino acids is shifted in comparison with the alpha amino acids and likely unable to fit properly in the binding pocket of the receptor.

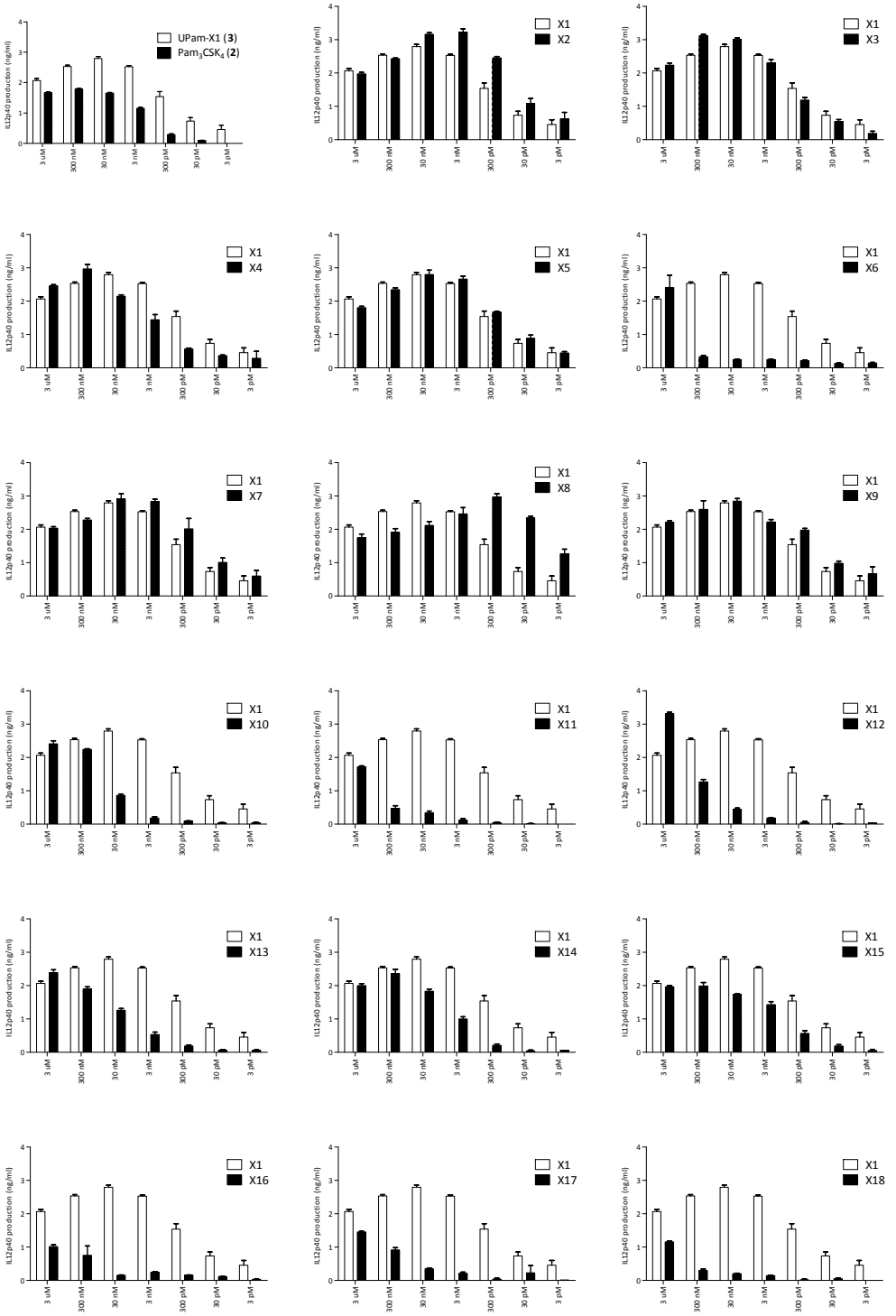


Figure 4. DC Maturation potency of **UPam-X1 – UPam-X18**.

The potency of the UPam-**Xn** library was further investigated by measuring the upregulation of the cell surface markers CD40, CD86 and MHC class II at 3 μM and 30 nM (*Figure 5 A – C*). Pam_3CSK_4 (**2**) was used as a reference. The potency of the compounds to upregulate CD40 on DCs followed the trend as observed in the DC maturation assay. Ligands with amino acids **X1**, **X2** - **X5**, and **X7** - **X9** show an increased amount of upregulation. In the upregulation of the cell surface marker CD86 and MHC class II a corroborated trend was observed. For all markers ligands with amino acids **X10**, **X13** – **X15** were found to have a comparable activity as **2** and a lower activity than UPam-**X1**. The derivatives with modifications **X6**, **X11**, **X12**, **X16** – **X18** were found to be inactive.

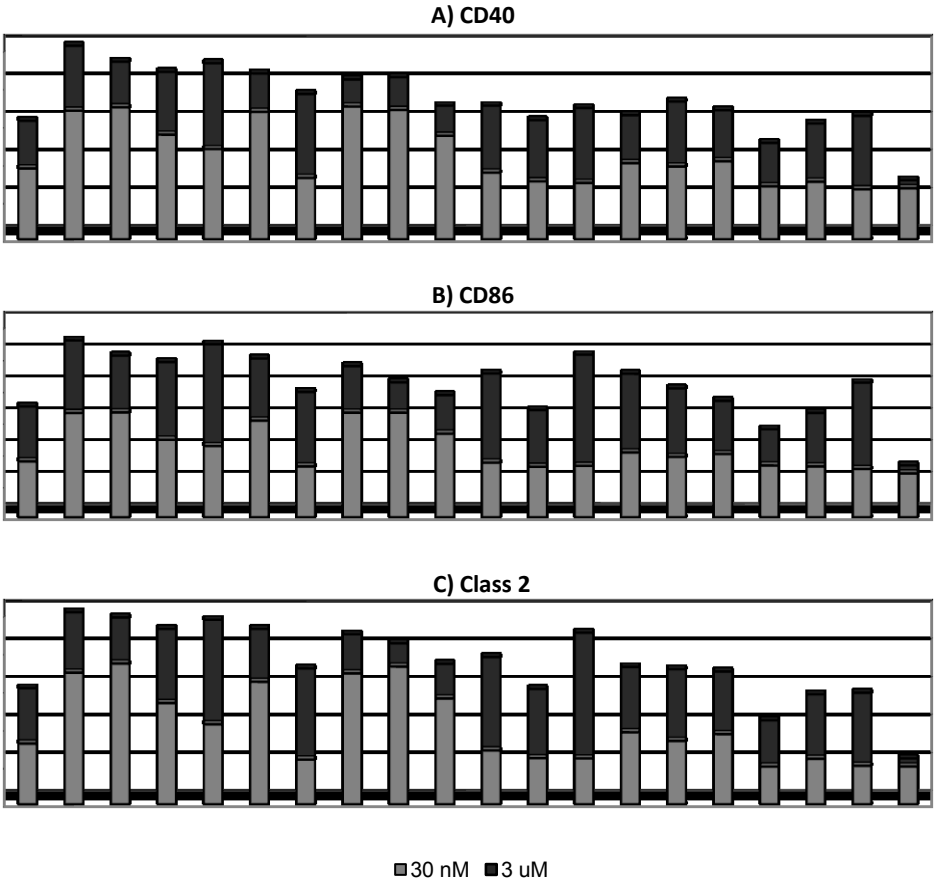


Figure 5. Cell surface markers upregulation of compounds UPam-**X1** – UPam-**X18** and Pam_3CSK_4 (**2**).

6.3 Synthesis and immunological evaluation of UPam-antigen conjugate

Previously it was shown that conjugate Pam_3CSK_4-$DEVA_5K$ (**8**), comprising the TLR2 ligand Pam_3CSK_4 covalently linked to antigenic peptide $DEVA_5K$, exhibited an improved immunological profile in comparison with a mixture of the individual components (*Figure 6*). To investigate the influence of UPam-**Xn** ligands, UPam-antigen conjugate **7**, in which Upam-**X1** is covalently linked to the peptide $DEVA_5K$, was synthesized using same approach as described for the UPam library. Conjugate **7** was obtained in 8% overall yield.

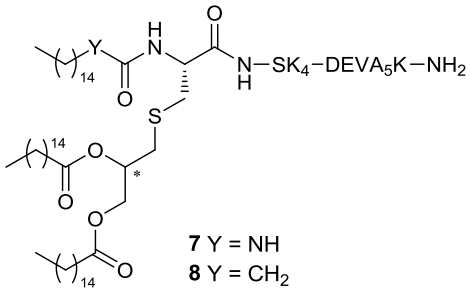


Figure 6. UPam-antigen conjugate **7** and Pam_3C-antigen conjugate **8**.

To monitor the immunological activity of conjugate **7** DCs maturation and antigen presentation were tested. The maturation is measured in D1 DCs in which IL-12p40 production is measured. Antigen uptake was measured in a T-cell hybridoma assay where the level of antigen presentation is measured spectrophotometrically (OD590nm).
In the DC maturation assay, conjugate **7** was tested in comparison to conjugate **8** and the free ligands **2** (Pam_3CSK_4) and UPam-**X1** as revealed in *Figure 7*. UPam based conjugate **7** is detectably more active than parent construct **8**.

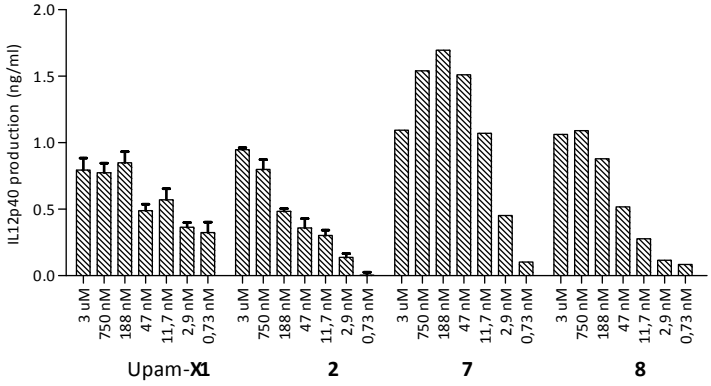


Figure 7. DC maturation induced by conjugates **7** and **8** and ligands **2** and Upam-**X1**.

In the MHC Class I antigen presentation assay conjugate **7** was tested in comparison to parent conjugate **8** and unconjugated peptide **9** ($DEVA_5K$) (*Figure 8*) and this assay shows that conjugates **7** and **8** are equally capable in inducing an enhancing antigen presentation by dendritic cells.

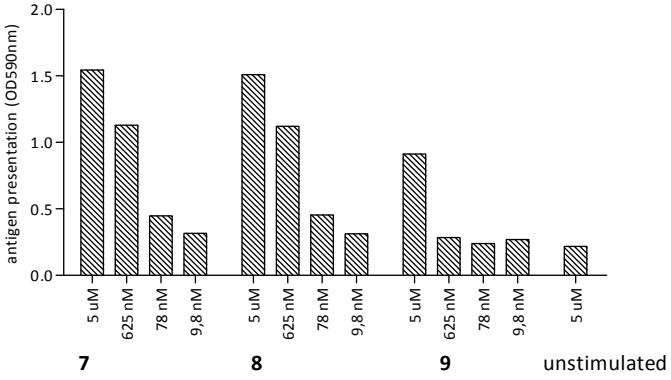


Figure 8. Antigen presentation of conjugates **7** and **8** and peptide **9**.

6.4 Conclusion

A novel analogue of Pam_3Cys, termed UPam, containing an urea moiety as a substitute for the cysteine amide bond was designed on the basis of the X-ray structure of the TLR1/TLR2 dimer co-crystallized with a Pam_3Cys based ligand. The new TLR2-ligand (UPam-**X1**) proved to be more potent than the original Pam_3Cys ligand. Modification of the serine in the UPam based ligand led to several ligands (UPam-**X2**, UPam-**X8**, UPam-**X5** and UPam-**X7**) with slightly improved immunostimulatory activity. In addition, conjugation of UPam-**X1** with an antigenic peptide gave conjugate **7**, having slightly higher maturation potency and an equal ability to induce antigen presentation in comparison with prototype conjugate **8**.

6.5 Experimental section

All reagents and solvents used in the solid phase peptide synthesis were purchased from Bachem and Biosolve and used as received. Palmitoyl-Cys((RS)-2,3-di(palimitoyloxy)-propyl)-OH was purchased from Bachem, Fmoc-amino acids from Novabiochem and HATU and tetradecyl isocyanate from Tebu Bio. Tentagel based resins were ordered from Rapp Polymere.

H-Lys-Lys-Lys-Lys-NH_2

Peptide synthesis was performed on a 1 mmol scale using an ABI 433A automated instrument applying Fmoc based protocol starting form Rink Amide S Tentagel (loading 0.26 mmol/g). The resin, after final Fmoc deprotection, was washed with NMP and DCM and dried. On 23 µmol of the resin was performed a HCTU coupling with Fmoc-Phe-OH to increase retention for LCMS analysis. This aliquot was transferred to a tube and treated for 104 minutes with a cleavage cocktail TFA/TIS/H_2O (95/2.5/2.5). The solution was filtered and the peptide was precipitated out of solution with Et_2O (50 mL) and the resin was washed with neat TFA. The Et_2O was centrifuged, removed and the precipitated was washed with Et_2O. The washing was repeated (3x). After centrifugation the Et_2O was removed and the precipitate of Fmoc-FKKKK-NH_2 was air dried and dissolved in MeCN:H_2O:tBuOH (1:1:1). LC/MS: Rt = 8.40 min (C_{18} Alltima, 0 - 50% MeCN, 15 min run); ESI-MS: *m/z* 899.55 $[M+H]^+$.

General procedure coupling amino acids X1- X18 on serine-position, Library 1.

One mmol H-Lys-Lys-Lys-Lys preloaded resin was suspended in DCM : NMP (30 : 15 mL) in total volume of 50 mL to pipette 1 mL portions of 20 µmol resin, followed by draining the syringes. The Fmoc amino acids X1-X18 applied in the synthesis:

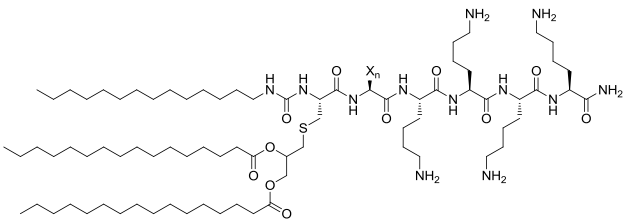
X_n		X_n		X_n	
X1	*Fmoc-(S)-Ser-OH*	*X7*	*Fmoc-Pra-OH*	*X13*	*Fmoc-Thi-OH*
X2	*Fmoc-Abu-OH*	*X8*	*Fmoc-Dab(Boc)-OH*	*X14*	*Fmoc-Thienylglycine*
X3	*Fmoc-Nva-OH*	*X9*	*Fmoc-Asn(Trt)-OH*	*X15*	*Fmoc-Gly-OH*
X4	*Fmoc-Nle-OH*	*X10*	*Fmoc-(R)-Cys(Trt)-OH*	*X16*	*Fmoc-Bal-OH*
X5	*Fmoc-(S)-Agl-OH*	*X11*	*Fmoc-(S)-Cys(Trt)-OH*	*X17*	*Fmoc-Bly-OH*
X6	*Fmoc-(R)-Agl-OH*	*X12*	*Fmoc-Pen(Trt)-OH*	*X18*	*Fmoc-Nal-OH*

Amino acids X1-X18 were pre-activated with a stock solution of 0.5 mL 0.22 M PyBOP in DCM:NMP (2:1) and 36.5 µL DiPEA and added to the 20 µmol resin in syringes labeled for X1 to X18. The resulting mixture was reacted for 1h by shaking followed by a NMP wash. The resin was capped 3 x 15 min with 1mL 0.5M Ac_2O : DiPEA (2:1) in NMP followed by a NMP and DCM wash. The final Fmoc-protection was removed in a 3 x 3 min cycle of 20% piperidine in NMP continued with an NMP and DCM wash.

General procedure coupling Fmoc-Cys((RS)-2,3-di(palimitoyloxy)-propyl)-OH

20 µmol tentagel S Ram resin loaded with NH_2-X_n-Lys-Lys-Lys-Lys was treated with a 0.5 mL stock solution of 0.18 M Fmoc-Cys((RS)-2,3-di(palimitoyloxy)-propyl)-OH in 0.22 M PyBOP in DCM : NMP (2 : 1). The resulting mixture was activated with 2 x 44 µmol DiPEA over 15 min. and reacted by shaking for 18h followed by NMP and DCM wash. The resin was swelled in DCM : NMP again and divided in portions of 10 µmol.

General procedure coupling tetradecyl isocyanate



The 10 µmol resin loaded with Fmoc-Cys((RS)-2,3-di(palimitoyloxy)-propyl)-X_n-Lys-Lys-Lys-Lys was swollen in DCM : NMP (1 : 1) and treated with 3 x 3 min 20% piperidine in NMP for Fmoc-deprotection. After thoroughly NMP wash the portions resin were suspended in 1 mL DCM : NMP (1 : 1) and treated with 25 µL tetradecyl isocyanate. The mixture was shaken for 18, washed with NMP and DCM and air dried. The resin was treated for 104 minutes with a cleavage cocktail of 95% TFA, 2.5% TIS and 2.5% H_2O. The solution was filtered and precipitated with Et_2O (50 mL) and stored at -20^{0}C for 18h. The Et_2O was centrifuged, removed and the precipitated was dissolved by sonification in 1 mL MeCN : H_2O : tBuOH (1 : 1 :1). A small amount (50 µL) was used for LCMS analysis (Vidac C_4 column). Obtained sequences were diluted with another 0.5 mL MeCN : H_2O : tBuOH (1 : 1 : 1) and were purified on a semiprep Vidac C_4 column (10 x 250 mm, 5 µm particle size, flow 5.0 mL/min, 60 - 100% MeCN.).

UPam-X1; ***1-tetradecyl-urea-Cys((RS)-2,3-di(palimitoyloxy)-propyl)-Ser-Lys-Lys-Lys-Lys-NH_2***
0.89 mg (0.59 µmol, 6%); LC/MS: Rt = 8.23 min (Vidac C_4, 50 - 90% MeCN, 15 min run); ESI-MS: *m/z* 1510.2 $[M+H]^+$; HRMS Calcd for $[C_{80}H_{156}N_{12}O_{12}S + H]^+$ 1510.17592, found 1510.17670.

UPam-X2; ***1-tetradecyl-urea-Cys((RS)-2,3-di(palimitoyloxy)-propyl)-Abu-Lys-Lys-Lys-Lys-NH_2***
3.22 mg (2.13 µmol, 21%); LC/MS: Rt = 8.31 min (Vidac C_4, 50 - 90% MeCN, 15 min run); ESI-MS: *m/z* 1508.2 $[M+H]^+$; HRMS Calcd for $[C_{81}H_{158}N_{12}O_{11}S + H]^+$ 1508.19665, found 1508.19725.

UPam-X3; ***1-tetradecyl-urea-Cys((RS)-2,3-di(palimitoyloxy)-propyl)-Nva-Lys-Lys-Lys-Lys-NH_2***
3.65 mg (2.40 µmol, 24%); LC/MS: Rt = 8.32 min (Vidac C_4, 50 - 90% MeCN, 15 min run); ESI-MS: *m/z* 1522.2 $[M+H]^+$; HRMS Calcd for $[C_{82}H_{160}N_{12}O_{11}S + H]^+$ 1522.20230, found 1522.21619.

UPam-X4; ***1-tetradecyl-urea-Cys((RS)-2,3-di(palimitoyloxy)-propyl)-Nle-Lys-Lys-Lys-Lys-NH_2***
2.97 mg (1.93µmol, 19%); LC/MS: Rt = 8.48 min (Vidac C_4, 50 - 90% MeCN, 15 min run); ESI-MS: *m/z* 1536.2 $[M+H]^+$; HRMS Calcd for $[C_{83}H_{162}N_{12}O_{11}S + H]^+$ 1536.22795, found 1536.22914.

UPam-X5; ***1-tetradecyl-urea-Cys((RS)-2,3-di(palimitoyloxy)-propyl)-(S)-Agl-Lys-Lys-Lys-Lys-NH_2***
2.40 mg (1.58 µmol, 16%); LC/MS: Rt = 8.61 min (Vidac C_4, 50 - 90% MeCN, 15 min run); ESI-MS: *m/z* 1520.2 $[M+H]^+$; HRMS Calcd for $[C_{82}H_{158}N_{12}O_{11}S + H]^+$ 153, 1520.19665 found 1520.19748.

UPam-X6; ***1-tetradecyl-urea-Cys((RS)-2,3-di(palimitoyloxy)-propyl)-(R)-Agl-Lys-Lys-Lys-Lys-NH$_2$***
1.52 mg (1.0 µmol, 10%); LC/MS: Rt = 8.57 min (Vidac C_4, 50 - 90% MeCN, 15 min run); ESI-MS: *m/z* 1520.2 $[M+H]^+$; HRMS Calcd for $[C_{82}H_{158}N_{12}O_{11}S + H]^+$ 1520.19665 found 1520.19760.

UPam-X7; ***1-tetradecyl-urea-Cys((RS)-2,3-di(palimitoyloxy)-propyl)-Pra-Lys-Lys-Lys-Lys-NH$_2$***
4.10 mg (1.70 µmol, 27%); LC/MS: Rt = 8.22 min (Vidac C_4, 50 - 90% MeCN, 15 min run); ESI-MS: *m/z* 1518.2 $[M+H]^+$; HRMS Calcd for $[C_{82}H_{156}N_{12}O_{11}S + H]^+$ 1518.18100, found 1518.18214.

UPam-X8; ***1-tetradecyl-urea-Cys((RS)-2,3-di(palimitoyloxy)-propyl)-Dap-Lys-Lys-Lys-Lys-NH$_2$***
2.37 mg (1,56 µmol, 16%); LC/MS: Rt = 10.75 min (Vidac C_4, 50 - 90% MeCN, 15 min run); ESI-MS: *m/z* 1523.2 $[M+H]^+$; HRMS Calcd for $[C_{81}H_{159}N_{13}O_{11}S + H]^+$ 1523.20755, found 1523.21037.

UPam-X9; ***1-tetradecyl-urea-Cys((RS)-2,3-di(palimitoyloxy)-propyl)-Asn-Lys-Lys-Lys-Lys-NH$_2$***
2.12 mg (1.38 µmol, 14%); LC/MS: Rt = 10.82 min (C_4 Alltima, 10 - 90% MeCN, 15 min run); ESI-MS: *m/z* 1537.19 $[M+H]^+$; HRMS Calcd for $[C_{81}H_{157}N_{13}O_{12}S + H]^+$ 769.09705, found 769.09771.

UPam-X10; ***1-tetradecyl-urea-Cys((RS)-2,3-di(palimitoyloxy)-propyl)-(R)-Cys-Lys-Lys-Lys-Lys-NH$_2$***
2.68 mg (1.76 µmol, 18%); LC/MS: Rt = 8.48 min (Vidac C_4, 50 - 90% MeCN, 15 min run); ESI-MS: *m/z* 1526.2 $[M+H]^+$; HRMS Calcd for $[C_{80}H_{156}N_{12}O_{11}S_2 + H]^+$ 1526.15307, found 1526.15434.

UPam-X11**; 1-tetradecyl-urea-Cys((RS)-2,3-di(palimitoyloxy)-propyl)-(S)-Cys-Lys-Lys-Lys-Lys-NH$_2$**
4.04 mg (2.56 µmol, 26%); LC/MS: Rt = 8.36 min (Vidac C_4, 50 - 90% MeCN, 15 min run); ESI-MS: *m/z* 1526.2 $[M+H]^+$; HRMS Calcd for $[C_{80}H_{156}N_{12}O_{11}S_2 + H]^+$ 1526.15307, found 1526.15387.

UPam-X12; ***1-tetradecyl-urea-Cys((RS)-2,3-di(palimitoyloxy)-propyl)-Pen-Lys-Lys-Lys-Lys-NH$_2$***
2.84 mg (1.83 µmol, 18%); LC/MS: Rt = 8.53 min (Vidac C_4, 50 - 90% MeCN, 15 min run); ESI-MS: *m/z* 1555.19 $[M+H]^+$; HRMS Calcd for $[C_{82}H_{160}N_{12}O_{11}S_2 + H]^+$ 1554.18437, found 1554.18555.

UPam-X13; ***1-tetradecyl-urea-Cys((RS)-2,3-di(palimitoyloxy)-propyl)-Thi-Lys-Lys-Lys-Lys-NH$_2$***
2.06 mg (1.31 µmol, 13%); LC/MS: Rt = 8.78 min (Vidac C_4, 50 - 90% MeCN, 15 min run); ESI-MS: *m/z* 1576.2 $[M+H]^+$; HRMS Calcd for $[C_{84}H_{158}N_{12}O_{11}S_2 + H]^+$ 1576.16872, found 1576.16883.

UPam-X14; ***1-tetradecyl-urea-Cys((RS)-2,3-di(palimitoyloxy)-propyl)-Tgly-Lys-Lys-Lys-Lys-NH$_2$***
3.12 mg (2.00 µmol, 20%); LC/MS: Rt = 8.78 min (Vidac C_4, 50 - 90% MeCN, 15 min run); ESI-MS: *m/z* 1562.2 $[M+H]^+$; HRMS Calcd for $[C_{83}H_{156}N_{12}O_{11}S_2 + H]^+$ 1562.15307, found 1562.15408.

UPam-X15; ***1-tetradecyl-urea-Cys((RS)-2,3-di(palimitoyloxy)-propyl)-Gly-Lys-Lys-Lys-Lys-NH$_2$***
1.07 mg (0.72 µmol, 7%); LC/MS: Rt = 11.06 min (Vidac C_4, 50 - 90% MeCN, 15 min run); ESI-MS: *m/z* 1480.2 $[M+H]^+$; HRMS Calcd for $[C_{79}H_{154}N_{12}O_{11}S + H]^+$ 1480.16535, found 1480.16613.

UPam-X16; ***1-tetradecyl-urea-Cys((RS)-2,3-di(palimitoyloxy)-propyl)-Bal-Lys-Lys-Lys-Lys-NH$_2$***
3.76 mg (2.52 µmol, 25%); LC/MS: Rt = 8.61 min (Vidac C_4, 50 - 90% MeCN, 15 min run); ESI-MS: *m/z* 1494.2 $[M+H]^+$; HRMS Calcd for $[C_{80}H_{156}N_{12}O_{11}S + H]^+$ 1494.18100, found 1494.18130.

UPam-X17; ***1-tetradecyl-urea-Cys((RS)-2,3-di(palimitoyloxy)-propyl)-Bly-Lys-Lys-Lys-Lys-NH$_2$***
2.31 mg (1.48 µmol, 15%); LC/MS: Rt = 7.38 min (Vidac C_4, 50 - 90% MeCN, 15 min run); ESI-MS: *m/z* 1565.3 $[M+H]^+$; HRMS calcd for $[C_{84}H_{165}N_{13}O_{11}S + H]^+$ 1565.25450, found 1565.25377.

UPam-X18; ***1-tetradecyl-urea-Cys((RS)-2,3-di(palimitoyloxy)-propyl)-Nal-Lys-Lys-Lys-Lys-NH$_2$***
2.26 mg (1.39 µmol, 14%); LC/MS: Rt = 9.02 min (Vidac C_4, 50 - 90% MeCN, 15 min run); ESI-MS: *m/z* 1621.3 $[M + H]^+$; HRMS Calcd for $[C_{90}H_{162}N_{12}O_{11}S + H]^+$ 1621.23106, found 1621.23175.

UPamX1-DEVA_5K;
1-tetradecyl-urea-Cys((RS)-2,3-di(palimitoyloxy)-propyl)-Ser-Lys-Lys-Lys-Lys-Asp-Glu-Val-Ser-Gly-Leu-Glu-Gln-Leu-Glu-Ser-Ile-Ile-Asn-Phe-Glu-Lys-Leu-Ala-Ala-Ala-Ala-Ala-Lys-NH_2 (7)
6.71 mg (1.66 µmol, 8%); LC/MS: Rt = 9.82 min (Vidac C_4, 10 - 90% MeCN, 15 min run); ESI-MS: *m/z* 4039.50 $[M+H]^+$; HRMS Calcd for $[C_{192}H_{338}N_{40}O_{50}S + H]^{2+}$ 2020.25296, found 2020.24719.

Biological assays

Cell culture
For detailed information see the experimental section of Chapter 2.[18,19,20]

In vitro DC maturation assay
For detailed information see the experimental section of Chapter 2.

In vitro antigen presentation assay
For detailed information see the experimental section of Chapter 2.[20]

Flow cytometry analysis
For detailed information see the experimental section of Chapter 3.

5.6 References and notes

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